Studies on the mechanism of zymogen activation in blood coagulation

Promotor	Prof. Dr. H.C. Hemker
Copromotor	Prof. Dr. R.F.A. Zwaal
Referenten	Dr. J.H. Griffin
	Prof. Dr. L.L.M. van Deenen
	Prof. Dr. H.S.B. Struyker-Boudier

In this thesis work is presented that has been done in close cooperation with: Jan Rosing, Gerbrand van Dieyen, Coen Hemker, Rob Zwaal, Henk van Zutphen, Paul Comfurius, José Govers-Riemslag and last but certainly not least Truus Janssen-Claessen.

The manuscript was typed by Mrs. Helene Vermeer and Mrs. Barbara Geers.

Aan Geja en Bram voor
al die gemiste uren.

Murphy's Law:
If anything can go wrong it will
Sharon, T.M. (1979) *J.I.R.* 25, 23

'From now on I'm thinking only of me'
Major Danby replied indulgently with a
superior smile : 'But, Yossarian suppose
everyone felt that way'.
'Then', said Yossarian, 'I'd certainly be a
damned fool to feel any other way,
wouldn't I !?'
J. Heller Catch 22

Sturgeon's Revelation:
90% of everything is crud
Sharon, T.M. (1979) *J.I.R.* 25, 23

CONTENTS

CHAPTER 1

INTRODUCTION

Most people take it for granted that bleeding from a wound stops within a few minutes. However, history has provided us with some famous examples, like the Russian czar family, that this cannot be taken for granted at all.

The events responsible for the arrest of bleeding from a wound are called the haemostatic mechanism. Actually, thrombosis which can be regarded as a positive overshoot of the haemostatic mechanism is of much more medical importance than bleeding tendencies are. Bleeding disorders and thrombotic disorders together account for well over half of all deaths in western society. The importance of research on the haemostatic mechanism can therefore hardly be exaggerated.

Haemostasis is the result of a complex series of physiological and chemical events taking place in the blood. The history of research in this field dates back some 150 years. In 1835 Buchanan showed that the formation of the fibrin clot is caused by an enzyme demonstrable in the clot (1). Later work a.o. of Schmidt and Peeckelharing was adequately summarised by Morawitz (1910) in the formulation of the four factor hypothesis of coagulation. He postulated that the formation of a fibrin clot was the result of the interaction of four factors: prothrombin, fibrinogen, calcium ions and thromboplastin (2). It was thought that thromboplastin and calcium ions were necessary for the conversion of prothrombin into thrombin and that thrombin converted fibrinogen into fibrin.

Nowadays, it is known that coagulation is a much more complicated process. Blood coagulation is taken care of by proteins, called clotting factors, which are present in the blood. In a way this is an advantage because they are ready at hand when needed. However, it is clear that some means must exist to avoid blood clotting when not necessary. This is achieved by the fact that the clotting factors are present in the blood in a form without emzymatic activity (zymogen form). Coagulation thus results from the activation of these clotting factors.

Based on reactions and interactions between clotting factors known at that time Macfarlane (3) and Davie and Ratnoff (4) in 1964 independently put forward the so-called cascade mechanism for blood coagulation. A simplified representation of the blood coagulation

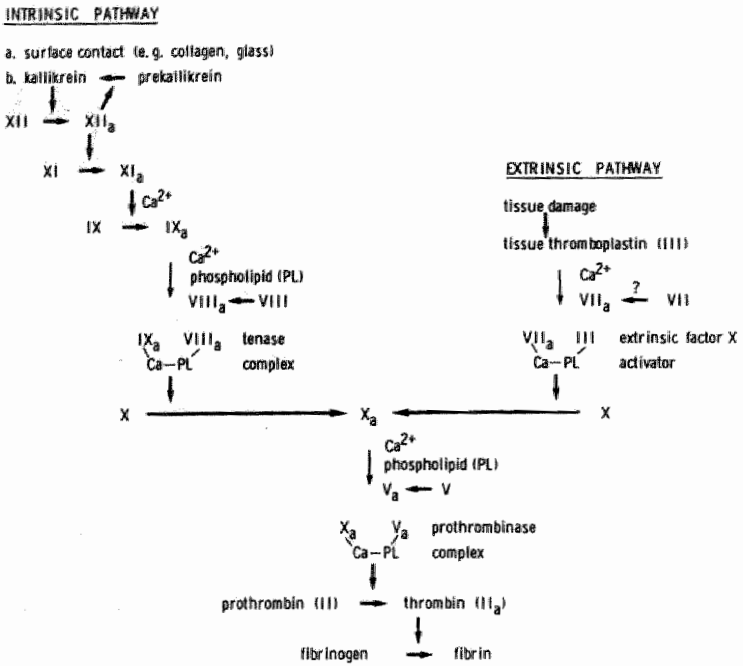


Fig. 1. Simplified representation of the blood coagulation cascade

Activated clotting factors are denoted by the subscript a. Note the similarity between the complexes in which phospholipid is involved: the intrinsic factor X activator, the extrinsic factor X activator and the prothrombinase complex. Taken from ref. 12.

cascade as it is nowadays understood is presented in Fig. 1.

Coagulation can be initiated in a number of ways. Depending upon initiation two different chains of events occur, i.e. the so-called intrinsic and extrinsic pathway of coagulation. Both pathways proceed via a series of consecutive activations of clotting factors present in the blood. Both the intrinsic and extrinsic pathway result in the activation of the zymogen factor X to the active form factor X_a. Factor X_a is the enzyme which together with factor V_a, phospholipid and calcium ions activates prothrombin to thrombin. Thrombin converts fibrinogen to fibrin and the fibrin network is formed.

A number of advantages for such an enzyme cascade can be recognised. First, the cascade mechanism has considerable potential for amplification. Only a small trigger signal is required to give an explosive burst of thrombin formation. For example a few molecules of

activated factor XII, resulting from the trigger, can activate hundreds of factor XI zymogens which in turn can activate some ten thousand factor IX zymogens. The plasma concentrations of the coagulation factors increase along the cascade (5) so efficient amplification can be achieved. A second advantage is the abundant number of possibilities for control. There is a number of positive and negative feedback control steps. For example in contact activation there is considerable amplification by the fact that factor XII_a can activate prekallikrein to kallikrein which in turn can activate the factor XII zymogen (6). A second example of a positive feedback mechanism is the activation of factors V and VIII by trace amounts of thrombin (for reviews see ref. 7,8). The activated clotting factors V_a and $VIII_a$, which are devoid of enzymatic activity, strongly enhance the rate of activation of respectively prothrombin and factor X. A possible negative feedback control mechanism is the inactivation of both factors V_a and $VIII_a$ by larger amounts of thrombin (7,9). Another example of negative control is the inactivation of various activated clotting factors by inhibitors present in the blood, e.g. antithrombin III and α_2-macroglobulin. Finally,biological control is exerted by the liver which rapidly removes activated clotting factors (rather than zymogens) from the circulation (10).

All enzymes participating in the coagulation cascade belong to the class of serine proteases. Factors IX_a, X_a and thrombin show considerable homology with the pancreatic serine proteases trypsin and chymotrypsin suggesting a common ancestry and comparable function. Trypsin and chymotrypsin are also enzymes which are normally present in the zymogen form and have to be activated by limited proteolysis. For an excellent book on the relationship between enzyme structure, molecular mechanism and kinetics of pancreatic serine proteases the reader is referred to ref. 11. Since clotting factors are homologous to these enzymes it is expected that their structure and the reactions in which they are involved will show a high degree of similarity with the reactions catalysed by trypsin and chymotrypsin (see ref. 9)

Blood coagulation is only a part of the haemostatic mechanism. Over the past 75 years through clinical, physiological and biochemical research a picture of this mechanism has emerged which shows that vessel wall, thrombocytes and plasma clotting factors cooperate in a complex way to achieve haemostasis. The haemostatic mechanism as a

whole is still not fully understood and at regular intervals new aspects are reported which have to be included in the overall picture.

At the risk of oversimplification it can be outlined as follows. When a lesion occurs in the vessel wall, either by injury or by the development of an atherosclerotic plaque, blood platelets adhere almost immediately to the subendothelial structure which has become exposed. The adhered platelets release substances which stimulate the aggregation of other platelets. At the same time the blood coagulation cascade is triggered. The thrombin formed has two major functions. It strongly activates platelets resulting in the formation of platelet aggregates and it converts fibrinogen into fibrin. The fibrin network resulting from the clotting stabilises the platelet plug. This completes the haemostatic process leading to a permanent arrest of bleeding.

The two processes, the formation of the platelet plug and the fibrin clot are closely interrelated at many stages. For example the platelets provide the phospholipid surface necessary in some reactions in the clotting cascade. The work presented in this thesis concerns the interactions of phospholipid and coagulation factors. Its purpose is to study in detail one of the parts of the haemostatic process in the hope that eventually enough of the completeprocess will be known to enable adequate intervention in hyper- and hypocoagulant diseased states

The reactions that we investigated show a peculiar feature which has not been discussed yet. It was already mentioned that clotting factors are in many aspect similar to the pancreatic serine proteases. However, activation of clotting factors is in general more complex than the activation of the zymogens from the pancreatic system. In many reactions of the coagulation cascade clotting factors are not activated by single enzymes, but by complexes in which the action of the enzyme on the substrate is stimulated by other components (cf. Fig. 1). Phospholipids participate in a number of these so-called multi-component enzyme systems. These are prothrombin activation and factor X activation both via the extrinsic and intrinsic pathway. The enzyme complexes in these three reactions have a similar composition. Apart from the enzyme, necessary to activate the substrate, they consist of calcium ions, phospholipid and a protein co-factor. The function of these accessory components in these complexes appears also alike. In prothrombin and factor X activation the rate of acti-

vation by the enzyme is enormously stimulated by the presence of calcium ions, phospholipid and the accessory protein. The mechanism via which these rate enhancements occur is still not fully understood. In chapter 2 further background information is given pertinent to the understanding of these reactions.

Phospholipid bilayers must fulfill a number of requirements in order to have a stimulatory effect in these reactions. The physical properties rather than the chemical properties of the phospholipid bilayer are important in this aspect. It is well established that the phospholipid bilayer must have a net negative charge in order to stimulate coagulation. Chapter 3 deals with the question whether phospholipid bilayers in the liquid crystalline phase show a different procoagulant activity than phospholipid bilayers in the solid gel phase.

Coagulation is a process which is tuned to produce an explosive burst of thrombin formation when needed. Control of this process is provided by a number of positive and negative feedback loops. To be able to appreciate the contribution of the different reactions to the overall reaction it is of vital importance to investigate the kinetics of the reactions involved. When the kinetic parameters of all the reactions of the clotting cascade are known it will be possible to evaluate their relative importance to clot formation in vivo. Quantitative study of these reactions has only become possible in the last few years with the elaboration of purification methods of the proteins involved and has been enormously facilitated by the appearance of a number of good chromogenic substrates for activated clotting factors. Chapter 4 is completely devoted to a kinetic analysis of prothrombin activation by factor X_a and to the effect of calcium ions, phospholipid and factor V_a on the kinetic parameters of prothrombin activation. Chapter 5 deals with a comparable kinetic analysis of factor X activation by factor IX_a in the presence and/or absence of calcium ions, phospholipid and factor $VIII_a$.

In order to be able to quantitate reaction rates it is necessary that the amounts of enzyme, substrate and product can be expressed on a molar basis. For factor X_a, thrombin, factor X and prothrombin this is readily possible. In chapter 6 it is shown that factor IX_a can be quantitated on a molar basis by active site titration of the enzyme with p-nitrophenyl-p'-guanidinobenzoate (p-NPGB).

The data presented in chapters 3, 4 and 5 all concern the role of phospholipid in coagulation. The reactions involved are thought to

take place on the surface provided by the phospholipids. However, exact binding parameters of the protein-phospholipid complexes under our circumstances are not known. In chapter 7, a simple and rapid method for the measurement of binding of factor X to the phospholipid surface is presented which may help to establish how the phospholipid surface mediates factor X activation.

The effects of accessory components on the kinetic parameters of prothrombin and factor X activation reported in chapters 4 and 5 have consequences for their mode of action in the zymogen activation. A general discussion on the role of phospholipid and factor V_a and factor VIII in the mechanism of activation of prothrombin and factor X is presented in chapter 8.

REFERENCES

1. Buchanan, A. (1835) *Med.Gaz.* 18, 50
2. Morawitz, P. (1905) *Ergebnisse der Physiologie Biologischen Chemie und Experimental Pharmakologie* 4, 307
3. Macfarlane, R.G. (1964) *Nature (London)* 202, 498-499
4. Davie, E.D. and Ratnoff, O.D. (1964) *Science* 145, 1310-1312
5. Davie, E.W. and Kirby, E.P. (1973) In Current Topics in Cell Regulation (Honecker, B.L. and Stadtman, E.R., Eds.) Vol. 7, 51-86, Acad.Press, New York
6. Griffin, J.H. and Cochrane, C.G. (1979) In Seminars in Thrombosis and Haemostasis. Vol.V, No. 4, p. 254-273
7. Davie, E.W. and Fujikawa, K. (1975) *Ann.Rev.Biochem.* 44,799-829
8. Davie, E.W., Fujikawa, K., Kurachi, K. and Kisiel, W. (1979) *Adv.Enzymol.* 48, 277-318
9. Silverberg, S.A. and Nemerson, Y. (1975) *Biochemistry* 14, 2636-2644
10. Deykin, D., Cochior, F., De Camp, G. and Lopez, A. (1968) *Am.J.Physiol.* 214, 414-419
11. Fersht, A. (1977) Enzymes, Structure and Mechanism. W.H.Freeman and Company Ltd., Reading and San Francisco
12. Zwaal, R.F.A. (1978) *Biochim.Biophys.Acta* 515,163-205

CHAPTER 2

LITERATURE

It is quite a hopeless task to try and give an extensive review of the literature on coagulation. The work presented in this thesis will contribute to the present understanding of prothrombin activation, factor X activation via the intrinsic pathway and, by extension, extrinsic factor X activation. Therefore, I will restrict myself to the literature pertinent to the understanding of these reactions. This chapter is divided into paragraphs dealing with:

2.1: prothrombin activation by the prothrombinase complex (i.e. factor X_a, factor V_a, Ca^{2+} and phospholipid)

2.2: factor X activation both by the intrinsic factor X activator (i.e. factor IX_a, factor VIII, Ca^{2+} and phospholipid) and by the extrinsic factor X activator (i.e. factor VII_a, Ca^{2+} and tissue thromboplastin)

2.3: phospholipid involvement in blood coagulation.

For reviews on coagulation, the reader is referred to references 1-3, for a review on phospholipid involvement in coagulation to reference 4, and for reviews on phospholipids and phospholipid phase transitions to references 5 and 6.

2.1 Prothrombin activation

The activation of prothrombin provides the best documented example of zymogen activation in blood coagulation. Bovine prothrombin (Fig. 1) is a glycoprotein of 72,000 M.W. containing 11% carbohydrate (N.B. human prothrombin differs from bovine prothrombin only in minor details). Bovine prothrombin consists of one single polypeptide backbone of 582 amino acid residues. It is one of the so-called vitamin K dependent clotting factors (like factors X, VII, IX, and protein C). These factors possess a number of unusual amino acid residues i.e. γ-carboxyglutamic acid, which provides Ca^{2+}-binding sites. Prothrombin is the zymogen which can be activated to thrombin through limited proteolysis. Several proteolytic enzymes can bring about this activation but under physiological conditions the serine protease factor X_a is the activating enzyme.

In the past 25 years many papers appeared in the literature describing the detection and isolation of partial proteolysis products of prothrombin activation (for a review see ref. 3). This finally resulted in a series of excellent papers by the group of Jackson (7-12) which

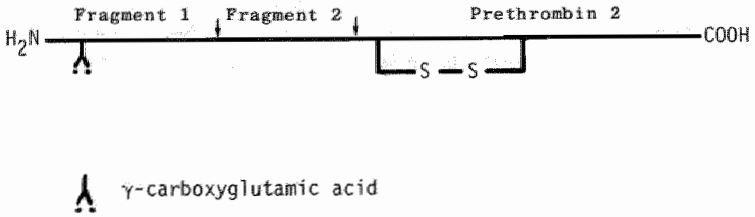


Fig. 1. Schematic representation of the prothrombin molecule

Fragment 1 is the region which contains the 10 γ-carboxyglutamic acid residues. Fragment 2 presumably has binding places for factor V. Prethrombin 2 is the immediate precursor of thrombin.

completed the description of the sites of peptide-bond splitting and order of bond cleavage during prothrombin activation. The activation of prothrombin into thrombin by factor X_a is thought to result from the two-step mechanism as depicted in Fig. 2.

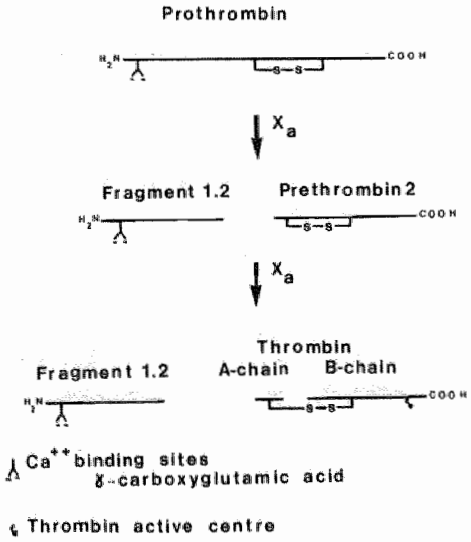


Fig. 2. Schematic representation of prothrombin activation by factor X_a

Factor X_a first cleaves off fragment 1.2. Prethrombin 2 is then cleaved to result in thrombin and the active site comes free. Fragment 1.2 can be cleaved by thrombin to result in fragment 1 and fragment 2.

The first step is the cleavage of an arginine-threonine peptide bond resulting in an activation peptide (fragment 1.2) and an intermediate (prethrombin 2). Thrombin activity appears after the cleavage of an arginine-isoleucine bond in this intermediate. Thrombin is a two-chain molecule held together by a disulfide bridge. The light chain contains 49 amino acid residues. The active site is located in the

heavy chain, which contains 259 amino acid residues.

Although this activation mechanism is the most plausible it must be emphasized that there are examples of other mechanisms. First, prothrombin can be activated by the formation of a one-to-one complex with staphylocoagulase, an exoprotein produced by certain strains of Staphylococcus aureus (13). Without the occurrence of peptide bond cleavage, staphylocoagulase presumably alters the conformation of prothrombin resulting in the formation of an active centre which is comparable to that of thrombin (13) but for its action on platelets and the factors V and VIII. Secondly, the occurrence of an intermediate (i.e. meizothrombin) has been reported in the activation of an abnormal prothrombin (14). Meizothrombin results from prothrombin by the cleavage of the peptide bond in the prethrombin 2 region and has proteolytic activity. This intermediate also occurs when prothrombin is activated by an enzyme from Echis Carinatus (15). Thus, the order of bond cleavage in prothrombin activation by factor X_a may not be compulsory.

In vitro, the prothrombin molecule can be cleaved by thrombin resulting in fragment 1 and prethrombin 1. Thrombin can also split fragment 1.2 into fragments 1 and 2. The possible regulatory function of this feedback reaction is, however, doubtful since it has been reported that this cleavage does not occur in whole blood (16).

It has been shown (see ref. 3) that additional components are essential for prothrombin activation during blood coagulation. It is now generally accepted that Ca^{2+}, phospholipid and factor V_a are required for prothrombin activation under physiological conditions. The factors X_a, V_a and prothrombin have to be adsorbed on the phospholipid surface in order to acquire efficient interaction (see ref. 3). Both prothrombin and factor X_a bind to the phospholipid surface via calcium bridges between the γ-carboxyglutamic acid residues present in these proteins and polar head groups of the phospholipid molecules (17,18),while hydrophobic interactions presumably play an important role in the binding of factor V_a (19,20). Thus the complex can be depicted as shown in Fig. 3.

It is well established that Ca^{2+}, phospholipid and factor V_a function as co-factors of the enzyme X_a bringing about a considerable enhancement of the relative rates of prothrombin activation (12,21). Not much is known, however, about the mechanism responsible for the enhancement of the rate of prothrombin activation by factor V_a and

phospholipid. Esmon and Jackson did not find evidence for a change sites and order of bond cleavage when the prothrombin molecule is activated by different combinations of the components of the prothrombinase complex (10).

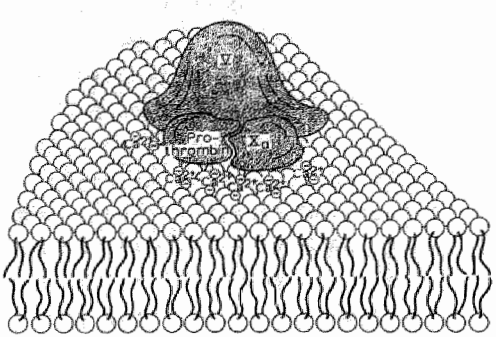


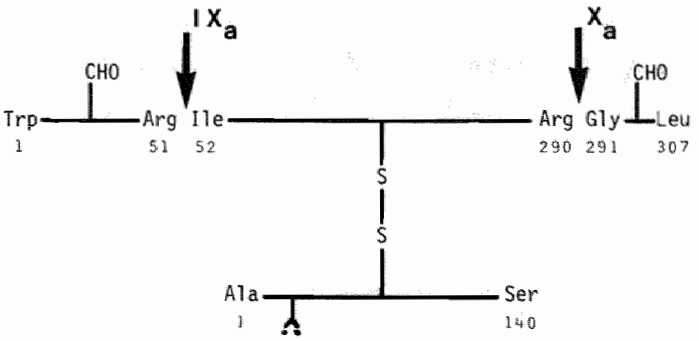
Fig. 3. Schematic model for the prothrombinase-prothrombin complex (Taken from ref.4).

2.2 Factor X activation

Bovine factor X (see Fig. 4) is a glycoprotein of MW 55,000, contai ing 10% carbohydrate, which is composed of a light and a heavy chai held together by a disulfide bridge (again the human material is hardly different). The light chain contains 140 amino acid residues 12 of which are γ-carboxyglutamic acid residues (22). The heavy cha consists of 307 amino acid residues and contains the active centre which is exposed upon splitting an arginyl-isoleucine bond, thus releasing an activation peptide containing 51 amino acid residues from the heavy chain N-terminal (23,24).

Upon purification two different forms of factor X, called factor X_1 and factor X_2, are found which differ in elution pattern on DEAE-Sephadex A-50 (25). Up till today, however, no other differences between these two forms have been reported.

Factor X can be activated both via the extrinsic and intrinsic pathway. It can also be activated by a protease from Russell's viper venom and by trypsin. All these activations occur through the cleavage of the same peptide bond resulting in the so-called αfactor X_a (26). There are several feedback reactions which may hamper a quantitative study of factor X activation. Jesty et al. (27,28) have shown that factor X_a can autocatalytically cleave a small peptide from the carboxy terminal end of the heavy chain of the zymogen resulting in the so-called βfactor X (see Fig. 4).



γ-carboxyglutamic acid residues

CHO carbohydrate

Fig. 4. Schematic representation of the bovine factor X molecule

The light chain contains 12 γ-carboxyglutamic acid residues. Factor X activation by factor IX_a results from the cleavage of the arginine-isoleucine bond as indicated. Factor X activation is also achieved by cleavage of this bond by factor VII_a, RVV-X or trypsin. Factor X can be split by factor X_a at the arginine-glycine peptide bond as indicated in the figure to result in βfactor X.

Also factor X_a can convert the zymogen into the active form by cleavage of the same arginyl-isoleucine bond. These reactions both require Ca^{2+} and phospholipid in order to result in detectable amounts of βfactor X and βfactor X_a.

2.2.1 The intrinsic factor X activator

Factor X can be activated via the intrinsic pathway by a complex which consists of factor IX_a, Ca^{2+}, phospholipid and factor VIII.

Factor IX_a is the activated form of the zymogen factor IX, a glycoprotein of MW 55,000 containing some 26% carbohydrate. It consists of a single polypeptide chain of 416 residues of wich 12 are γ-carboxyglutamic acid residues (29, see Fig. 5). In vivo, factor IX is activated by factor XI_a by the proteolytic cleavage of two peptide bonds. After the cleavage of the first peptide bond, an intermediate results which is inactive in coagulation and has no detectable esterase activity. Coagulant activity and esterase activity appear upon the cleavage of the second bond liberating an activation peptide of 9,000 MW (30). Factor IX can also be activated by RVV-X

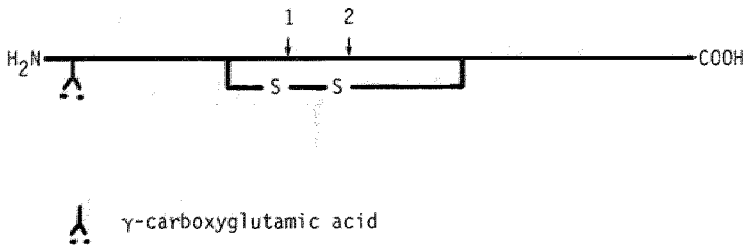


Fig. 5. Schematic representation of the bovine factor IX molecule

Factor IX can be activated by factor XI_a. First an arginine-alanine peptide bond is cleaved (indicated in the figure with 1), resulting in an inactive intermediate. Esterase activity appears as a result of the cleavage of an arginine-valine bond (indicated by 2). Factor IX_a is a two chain molecule of which the light chain contains 12 γ-carboxyglutamic acid residues. The active serine is located in the heavy chain.

by cleavage of the second bond resulting in the formation of an activated factor IX of the same MW as the zymogen (31). Recently, evidence has appeared that factor IX can also be activated by facto: VII_a and tissue thromboplastin (32, 33) explaining the earlier reports on interactions between the proteins involved in the extrin· sic pathway with the proteins involved in the intrinsic pathway (34,35,36). This latter finding suggests intriguing possibilities on the regulation of factor X activation during blood clotting.

It has been shown that the proteins involved in intrinsic factor X activation have to be adsorbed onto the phospholipid bilayer during the activation reaction (37-43). Not much is known about the roles of the individual components of the intrinsic factor X activator. Factor IX_a, since it is highly homologous to other serine proteases (29) will likely be the enzyme which activates factor X. Up till now, however, it has not been reported that factor IX_a, in the absence of the accessory components, i.e. factor VIII, phospholipid and Ca^{2+}, is capable of activating factor X. Hultin and Nemerson showed that a very low rate of factor X activation can be observed in the presence of Ca^{2+} and phospholipid (44), which is stimulated about 500 fold by the addition of thrombin activated factor VIII. Brown et al. (45) reported a K_m for factor X of 0.2 μM when factor X activation was measured in the presence of all accessory componen·

The role of factor VIII is still somewhat ambiguous. It has been reported many times that factor VIII has to be activated by trace amounts of thrombin in order to stimulate factor X activation (44,46-49). There is also evidence that factor VIII may be activated by factor X_a (47,49) and by factor IX_a (49). Of interest is the finding of Vehar and Davie (48,49) that thrombin activated factor VIII can be inhibited by diisopropylphosphorofluoridate (DFP). This may have consequences for the role of factor $VIII_a$ in the mechanism of factor X activation.

In conclusion, however, since intrinsic factor X activation is in many respects highly similar to prothrombin activation it seems plausible that factor $VIII_a$, Ca^{2+} and phospholipid act as co-factors of the enzyme factor IX_a analogous to the roles of factor V_a, Ca^{2+} and phospholipid in the prothrombinase complex.

2.2.2 The extrinsic factor X activator

Factor X activation via the extrinsic pathway is accomplished by a complex consisting of factor VII_a, Ca^{2+} and tissue thromboplastin. Factor VII_a is the enzyme in this complex and tissue thromboplastin and Ca^{2+} act as co-factors.

Factor VII_a is the activated form of the zymogen factor VII. Again this is a vitamin K dependent glycoprotein. It has a MW of 45,000. It is highly probable that factor VII contains some 10-12 γ-carboxy-glutamic acid residues (see ref. 1). It has been shown that the zymogen factor VII can activate factor X at a very low rate in the presence of tissue thromboplastin and Ca^{2+} (50). Factor X_a in its turn can activate factor VII and thus a strong feedback mechanism is provided (51,52). Factor XII_a and factor XI_a as well have been reported to be capable of the activation of factor VII (53,54). A possible regulatory mechanism can be recognised in the fact that both factor X_a and thrombin are also capable of inactivating factor VII_a. It will be clear that a detailed kinetic analysis of all these reactions is necessary to establish their relative importance.

The co-factor of factor VII_a in factor X activation, i.e. tissue thromboplastin,consists of phospholipid to which an insoluble membrane protein is bound (tissue factor). This complex strongly interacts with factor VII. Therefore, the roles of factor VII_a, Ca^{2+}, phospholipid and protein co-factor (tissue factor) seem completely identical to the roles of factor X_a, phospholipid and factor V_a in the

prothrombinase complex.

Silverberg et al. (55) reported a kinetic analysis of factor X activation by the extrinsic complex (see table I). In the absence of the protein cofactor, the catalytic efficiency (k_{cat}/K_m) of factor X activation is rather low. This is, however, dramatically increase by addition of tissue factor. The K_m is then below the factor X pla concentration and k_{cat} has reached a level which is normal among ser proteases. Therefore, it is easily recognised that without the prot co-factor bound to phospholipid there will be no physiological significant factor X_a formation during in vivo clotting.

Table I. Kinetic data for extrinsic factor X activation
Data taken from Silverberg et al.(55).

	With tissue factor	Without tissue factor
K_m (μM factor X)	0.45	4.87
V_{max} (pmol/min^{-1})	64	1.09
E (pmol)	0.93	46
k_{cat} (s^{-1})	1.15	3.95×10^{-4}
Coefficient of proteolytic efficiency $C_{P.E}$ (k_{cat}/K_m)	2.55	8.1×10^{-5}
Effect of tissue factor on $C_{P.E}$	31,500	1

2.3 Phospholipid involvement in blood coagulation

Phospholipids represent about 30% of the total mass of most mammali plasma membranes. The remaining 70% consist of membrane proteins, cholesterol and a small amount of glycolipids.

The general structure of a phospholipid molecule is shown in Fig. 6 The primary building block is a sn-glycerol-3-phosphoric acid molecu which results from esterification of a primary alcohol group of glycerol to phosphoric acid. The two remaining hydroxyl groups are esterified to fatty acids (R1 and R2). Phospholipids are classified according to their head group (X) which results from the esterification of an alcohol compound (X-OH) to the phosphoric acid group to form a phosphodiester. The three major classes are shown in the figure. Thus a phospholipid molecule consists of a polar region (the head group) and a non-polar region represented by the fatty

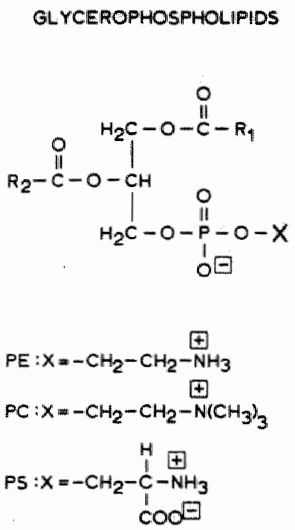


Fig. 6. Structure of a phospholipid molecule

Phospholipid molecules contain two fatty acyl chains resulting from the esterification of two adjacent alcohol groups of a sn-glycerol-3-phosphoric acid molecule to fatty acids (R1 and R2).

Phospholipids are classified according to their head group (X). The three major classes are shown. PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine. When the phosphoric acid group is not esterified (X=H) the resulting molecule is phosphatidic acid.

acid chains.

This is the reason why phospholipids spontaneously form concentric multilamellar structures (liposomes) when suspended in an aqueous environment (56). The head groups are in contact with the solvent and the non-polar region is excluded from the polar environment. Unilamellar vesicles can be obtained upon sonication of the liposome suspension (57, see Fig. 7). The phospholipids which are of prime

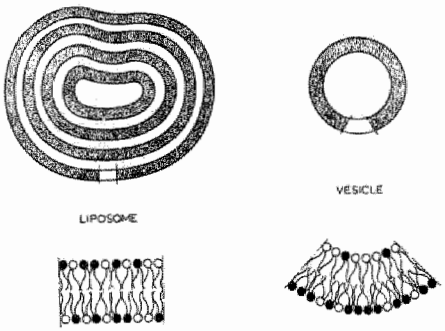


Fig. 7. Schematic representation of multilamellar liposomes and unilamellar vesicles

Due to the strong curvature, vesicles may be asymmetric both with respect to the number of PL molecules per monolayer and the distribution of PL over both layers when more than one PL class is present. Taken from ref. 4.

interest to the work presented in this thesis belong to the classes of phosphatidylcholine and phosphatidylserine. When mixtures of these

phospholipids are sonicated, vesicles with a diameter of about 250 Å are obtained (58).

The lipid bilayer is thought to be the structural framework of the membrane whereas the proteins perform most of the biochemical functions. Therefore, it is not surprising that a vast amount of literature has appeared concerning the physical characteristics of the lipid bilayer. Research in this field is carried out using sophisticated techniques like X-ray diffraction, N.M.R. and E.S.R., calorimetric studies etc. (for reviews, see 5, 6, 59-61). Most of this research is carried out using well-defined phospholipids. As a consequence much is known nowadays about the characteristics of the phospholipid bilayer. Much less is known about bilayers containing protein. When proteins which interact with the bilayer are present study of the physical characteristics becomes difficult. It appears that the physical state of the bilayer can change dramatically upon the introduction of proteins among the phospholipids and consequently great caution should be taken in extrapolation from the well defined systems to systems where proteins are present.

Lipid molecules in the bilayer are not confined to their position in the lattice (like atoms in a crystal are). Diffusion rates for phospholipid molecules in a bilayer in the liquid crystalline phase of 10^{-8} cm^2/s have been reported (62). This diffusion drops about ten-fold upon going to the gel state. Therefore, the lipid bilayer in the liquid crystalline state appears fluid-like whereas fluidity is drastically decreased in the solid gel state. The temperature at which this phase transition occurs is strongly dependent on the length and degree of unsaturation of the fatty acyl chains and on the nature of the head group. For example, 1,2-dimyristoyl-sn-glycero-3-phosphoserine (di-$C_{14:0}$-glycerophosphoserine or dimyristoylphosphatidylserine) has a phase transition temperature of about 35°C (63). 1,2-Dipalmitoyl-sn-glycero-3-phosphoserine (di-$C_{16:0}$-glycerophosphoserine or dipalmitoylphosphatidylserine) melts at 55°C (64) whereas 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (di-$C_{16:0}$-glycerophosphocholine or dipalmitoylphosphatidylcholine) has its transition temperature at 41°C (65). The miscibility properties of bilayers composed of different phospholipid molecules are dependent on the difference in chain length of the fatty acyl chains and on the nature of the head group of the different components. Mixing is close to ideal for two lipids with the same head group and differing only slightly in chain length. However, when the head groups differ,the

picture becomes more complicated. For example, dimyristoylphosphatidylcholine and dipalmitoylphosphatidylethanolamine show only partial miscibility both in the liquid crystalline and the gel phase (66). The nature of the physical state can be strongly influenced upon interaction with calcium ions. It has been reported that addition of calcium ions to bilayers composed of phosphatidylserine molecules results in an increase of the phase transition temperature of more than 100°C. Calcium ions do not appear to influence the physical state of phosphatidylcholine bilayers. As a consequence addition of calcium ions to bilayers consisting of a mixture of phosphatidylserine and phosphatidylcholine may result in phase separation (67-71).

Much of our present understanding of phospholipid involvement in blood coagulation has come from work on the prothrombinase complex. It is a pity, however, that most of this research is carried out with poorly defined phospholipid preparations such as egg-yolk phosphatidylcholine, beef-brain phosphatidylserine, inosithin and cephalin. These preparations contain a large variety of different molecular species and often contain impurities of other phospholipids as well.

Nevertheless, it is clear that mixtures of different phospholipid classes provide the best catalytic surface for coagulation. The physical rather than the chemical characteristics of this surface are important. A number of physical requirements must be met in order to observe procoagulant activity. It is well established that a correlation exists between net negative charge of the phospholipid bilayer and procoagulant activity. When no net negative charge is provided no procoagulant activity is observed. Thus, phosphatidylcholine vesicles have no clot promoting effect. The magnitude of the charge seems to be less critical. By varying the phosphatidylserine content in mixtures of egg-yolk phosphatidylcholine and bovine brain phosphatidylserine, Bull et al. (72) showed that there is an optimal procoagulant activity at about 30% phosphatidylserine content in the phospholipid bilayer.

Nowadays, it is accepted that the vitamin K dependent proteins bind to the phospholipid surface through calcium bridging between the γ-carboxyglutamic acid residues of these proteins and the negatively charged phospholipid head groups. Factors V and VIII interact with the bilayer presumably via more hydrophobic interactions (19,20; see ref. 4). The combination of the negatively charged serine head groups of phosphatidylserine together with the neutral head groups

of phosphatidylcholine is thus thought to be the most ideal phospl lipid bilayer. It has to be mentioned, however, that the interacti of factor V with the phospholipid bilayer does require the presenc of negatively charged phospholipids too (73).

Binding of the proteins, though being a prerequisite for procoagul activity does not necessarily mean that procoagulant activity appe Bull et al. (72) showed that mixtures of phosphatidic acid and pho phatidylcholine bind prothrombin very effectively. However, the pr coagulant activity is much less than for mixtures of phosphatidyl-serine and phosphatidylcholine.

There appears to be a correlation between procoagulant activity an the physical state of the bilayer. Sterzing and Barton (74) showed that hydrogenation of mixtures of phosphatidylcholine and phosphat dylserine decreases procoagulant activity. Hydrogenation of the fatty acyl chains shifted the phase transition from -14°C to 72°C. Subsequent addition of cholesterol, which is known to have a liquefying effect on the phospholipid bilayer in the gel phase, restored procoagulant activity (74). Therefore, it is likely that the phospholipid bilayer in the liquid crystalline phase shows optimal procoagulant activity.

It was mentioned already that most of this work has been done on the phospholipid involvement in the prothrombinase complex. Much less is known on the phospholipid requirement of the intrinsic and the extrinsic factor X activator. The requirements appear to be alike with respect to the requirement for negative charge. Wijn-gaards et al. (75) showed that the extrinsic factor X activator requires a phospholipid bilayer of moderate negative charge compose of a mixture of different phospholipid molecules. It has been re-ported that the phospholipid requirement for the intrinsic factor X activator may be different from the requirement for the prothrombin ase complex (76). Negative charge would still be needed but the composition of the bilayer showing optimal factor X activation was reported to differ from the optimal composition of the bilayer for prothrombin activation.

REFERENCES

1. Davie, E.W., Fujikawa, K., Kurachi, K. and Kisiel, W. (1979) *Adv.Enzymol.* 48, 227-318
2. Davie, E.W., Fujikawa, K. (1975) *Ann.Rev.Biochem.* 44, 799-829
3. Suttie, J.W. and Jackson, C.M. (1977) *Physiol.Rev.* 57, 1-70

4. Zwaal, R.F.A. (1978) *Biochim.Biophys.Acta* 515, 163-205
5. Lee, A.G. (1977) *Biochim.Biophys.Acta* 472, 237-281
6. Lee, A.G. (1977) *Biochim.Biophys.Acta* 472, 283-344
7. Owen, W.G., Esmon, C.T. and Jackson, C.M. (1974) *J.Biol.Chem.* 249, 594-605
8. Esmon, C.T., Owen, W.G. and Jackson, C.M. (1974) *J.Biol.Chem.* 249, 606-611
9. Esmon, C.T. and Jackson, C.M. (1974) *J.Biol.Chem.* 249,7782-7790
10. Esmon, C.T. and Jackson, C.M. (1974) *J.Biol.Chem.* 249,7791-7797
11. Esmon, C.T., Owen, W.G. and Jackson, C.M. (1974) *J.Biol.Chem.* 249, 7798-7807
12. Esmon, C.T., Owen, W.G. and Jackson, C.M. (1974) *J.Biol.Chem.* 249, 8045-8047
13. Bas, B.M., Muller, A.D. and Hemker, H.C. (1974) *Biochim.Biophys.Acta* 379, 164-171
14. Guillin, M.C., Bezeaud, A. and Ménaché, D. (1978) *Biochim.Biophys. Acta* 537, 160-168
15. Rabiet, M.J., Benarous, R., Labie, D. and Josso, F. (1978) *Abstr. XVIIth Congress Int.Soc.Haemat.* 862
16. Aronson, D.L., Stevan, L., Bull, A.P., Franza Jr, B.R. and Finlayson, J.S. (1977) *J.Clin.Invest.* 60, 1410-1418
17. Papahadjopoulos, D.P., Hougie, C. and Hanahan, D.J. (1962) *Proc.Soc. Exp. Biol. Med.* 111, 412-416
18. Barton, P.G. and Hanahan, D.J. (1969) *Biochim. Biophys.Acta* 187, 319-327
19. Kandall, C.L., Shohet, S.B., Akinbarni, T.K. and Colman, R.W. (1975) *Thrombos.Diathes. haemorrh.* 34, 256-270
20. Kahn, M.J.P. and Hemker, H.C. (1969) *Thrombos.Diathes.haemorrh.* 22, 417-430
21. Jobin, F. and Esnouf, M.P. (1967) *Biochem. J.* 102, 666-674
22. Enfield, D.L., Ericsson, L.H., Walsh, K.A., Neurath, H. and Titani, K. (1975) *Proc. Natl. Acad. Sci. USA* 72, 16-19
23. Titani, K., Fujikawa, K., Enfield, D.L., Ericsson, L.H., Walsh, K.A. and Neurath, H. (1975) *Proc. Natl. Acad. Sci. USA* 72, 3082-3086
24. Deutsch, E., Irsigler, K. and Lomoschitz, H. (1964) *Thrombos. Diathes. haemorrh.*12, 12-34
25. Jackson, C.M. and Hanahan, D.J. (1968) *Biochemistry* 7, 4506-4517
26. Fujikawa, K., Coan, M.H., Legaz, M.E. and Davie, E.W. (1974) *Biochemistry* 13, 5290-5294

27. Jesty, J. and Nemerson, Y. (1974) *J.Biol. Chem.* 243, 509-515
28. Jesty, J., Spencer, A.K. and Nemerson, Y. (1974) *J.Biol. Chem.* 249, 5614-5622
29. Katayama, K., Ericsson, L.H., Enfield, D.L., Walsh, K.A., Neurath, H., Davie, E.W. and Titani, K. (1979) *Proc. Natl. Acad. Sci. USA* 76, 4990-4994
30. Fujikawa, K., Legaz, M.E., Kato, H. and Davie, E.W. (1974) *Biochemistry* 13, 4508-4516
31. Lindquist, P.A., Fujikawa, K. and Davie, E.W. (1978) *J.Biol.Ch* 253, 1902-1909
32. Østerud, B. and Rapaport, S.I. (1977) *Proc. Natl.Acad. Sci. US.* 74, 5260-5264
33. Jesty, J. and Silverberg, S.A. (1979) *J.Biol. Chem.*254, 12337-12345
34. Josso, F. and Prou-Wartelle, O. (1965) *Thrombos.Diathes.haemor* *Suppl.*17, 35-44
35. Biggs, R. and Nossel, H.L. (1961) *Thrombos. Diathes. haemorrh.* 6, 1-4
36. Rapaport, S.I., Hjort, P.F., Patch, M.J. and Jeremic, M. (1966) *Scand.J.Haematol.* 3, 59-75
37. Schiffman, S., Rapaport, S.I. and Chong, M.M.Y. (1966) *Proc. Sc* *Exp. Biol. Med.*123, 736-744
38. Hougie, C., Denson, K.W.E. and Biggs, R. (1967) *Thrombos.Diathe* *haemorrh.*18, 211-222
39. Barton, P.G. (1967) *Nature* 215, 1508-1509
40. Hemker,H.C. and Kahn, M.J.P. (1967) *Nature* 215, 1201-1202
41. Østerud, B. and Rapaport, S.I. (1970) *Biochem. J.* 9, 1844-1861
42. Varadi, K. and Hemker, H.C. (1976) *Thrombos. Res.* 8, 303-317
43. Hemker,H.C., Kahn, M.J.P. and Devilee, P.P. (1970) *Thrombos. Diathes. haemorrh.*24, 214-223
44. Hultin, M.B. and Nemerson, Y. (1978) *Blood* 52, 928-940
45. Brown, J.E., Brugh, R.F. and Hougie, C. (1978) *Thrombos. Res.* 13, 893-900
46. Østerud, B., Rapaport, S.I., Schiffman, S. and Chong, M.M.Y. (1 *Br. J. Haematol.* 21, 643-660
47. Davie, E.W., Fujikawa, K., Legaz, M.E. and Kato, H. (1975) *Cold Spring Harbor Conference on Cell Proliferation* 2, 65-77
48. Vehar, G.A. and Davie, E.W. (1977)*Science* 197, 374-376
49. Vehar, G.A. and Davie, E.W. (1980) *Biochemistry* 19, 401-410
50. Nemerson, Y. Personal Communication

51. Radcliffe, R. and Nemerson, Y. (1975) *J.Biol.Chem.*250, 388-395
52. Radcliffe, R. and Nemerson, Y. (1976) *J.Biol.Chem.*251, 4797-4802
53. Kisiel, W., Fujikawa, K. and Davie, E.W. (1977) *Biochemistry* 16, 4189-4194
54. Altman, R. and Hemker, H.C. (1967) *Thrombos. Diathes. haemorrh.* 18, 525-531
55. Silverberg, S.A., Nemerson, Y. and Zur, M. (1977) *J.Biol.Chem.* 252, 8481-8488
56. Bangham, A.D., Standish, M.M. and Watkins, J.C. (1965) *J.Mol.Biol.* 13, 253-264
57. Huang, C. (1969) *Biochemistry* 8, 344-351
58. Berden,J.A.,Barker,R.W. and Radda,G.K. *Biochim.Biophys.Acta* 375 (1975) 186-208
59. Azzi, A. (1975) *Quart.Rev. Biophys.* 8, 237-316
60. Wennerström, H. and Lindblom, G. (1977) *Quart. Rev. Biophys.* 10, 67-97
61. Seelig, J. (1977) *Quart. Rev. Biophys.*10,353-419
62. Cullis, P.R. (1976) *FEBS Letters* 70, 223-228
63. Comfurius, P. and Zwaal, R.F.A. (1977) *Biochim. Biophys. Acta* 488, 36-42
64. Mac Donald, R.C., Simon, S.A. and Bear, E. (1976) *Biochemistry* 15, 885-891
65. Ladbrooke, B.D. and Chapman, D. (1969) *Chem. Phys. Lipids* 3, 304-367
66. Blume, A. and Ackerman, T. (1974) *FEBS Letters* 43, 71-74
67. Ohnishi, S.I. and Ito, T. (1973) *Biochem. Biophys. Res. Commun.* 51, 132-138
68. Ohnishi, S.I. and Ito, T. (1974) *Biochemistry* 13, 881-887
69. Ito, I. and Ohnishi, S.I. (1974) *Biochim. Biophys. Acta* 352, 29-37
70. Papahadjopoulos, D., Poste, G., Schaeffer, B.E. and Vail, W.J. (1974) *Biochim. Biophys. Acta* 352, 10-28
71. Lee, A.G. (1975) *Biochim. Biophys. Acta* 413, 11-23
72. Bull, R.K., Jevons, S. and Barton, P.G. (1972) *J.Biol.Chem.* 247, 2747-2754
73. Subbaiah, P.V., Bajwa, S.S., Smith, C.M. and Hanahan, D.J. (1976) *Biochim. Biophys. Acta* 444, 131-146
74. Sterzing, P.R. and Barton, P.G. (1973) *Chem. Phys. Lipids* 10, 137-148

75. Wijngaards, G., van Deenen, L.L.M. and Hemker, H.C. (1977) *Biochim. Biophys. Acta* 488, 161-171

76. Walsh, P.N. (1978) *Br. J. Haematol.* 40, 311-331.

Eur. J. Biochem. 95, 449–457 (1979)

Lipid Phase Transitions and Procoagulant Activity

Guido TANS, Henk van ZUTPHEN, Paul COMFURIUS, H. Coenraad HEMKER, and Robert F. A. ZWAAL

Department of Biochemistry, Biomedical Centre, State University of Limburg, Maastricht

(Received November 30, 1978)

1. The relation between acyl chain fluidity of synthetic phosphatidylserine/phosphatidylcholine mixtures and their activity in the prothrombinase complex has been investigated using differential scanning calorimetry and a one-stage prothrombinase assay.

2. Mixtures of dioleoylphosphatidylserine and dioleoylphosphatidylcholine which are liquid crystalline at physiological temperature proved to be considerably more active in coagulation than mixtures of dipalmitoylphosphatidylserine and dipalmitoylphosphatidylcholine which are in the gel phase, while this difference disappears completely upon addition of cholesterol. Varying the proportion of phosphatidylserine from 30 to 70 mol/100 mol has no significant influence on the coagulant behaviour.

3. The procoagulant activity of mixtures composed of dimyristoylphosphatidylserine and dipalmitoylphosphatidylcholine was compared with the phase diagram constructed from the calorimetric data. Going through the phase transition to the liquid state was accompanied by a sharp increase of clot-promoting activity.

4. The phospholipid concentration which exhibited maximal coagulant activity appeared to be strongly dependent on the miscibility properties of the lipids. Solid/solid immiscibility was observed both with mixtures of the two dimyristoyl compounds and the dipalmitoyl compounds, and a 5–10-fold increase in the lipid concentration required for maximal coagulant activity was observed below the phase transition. This shift was found to be absent with mixtures of the dioleoyl species or with dimyristoylphosphatidylserine/dipalmitoylphosphatidylcholine both of which showed essentially complete miscibility in both phases.

Phospholipids play a crucial role in blood coagulation by specifically accelerating some of the reaction steps leading to clot formation [1–4]. The final lipid-activated step in the coagulation cascade is the conversion of prothrombin into thrombin by the prothrombinase complex, which is composed of factor X_a, factor V, Ca^{2+} and phospholipids (see for recent review [5]). Although there is conclusive evidence that a negative surface charge of the phospholipids is required for coagulant activity [6–8], neither the magnitude of the charge nor the charge density alone are sufficient to account for the ability of the lipids to form a procoagulant surface. Significantly, it has been shown that the clot-promoting activity of synthetic disaturated phosphatidylserines, when mixed with natural phosphatidylethanolamine, is maximal with dimyristoylphosphatidylserine, whereas the activity decreases using species with longer or shorter hydrocarbon chains [9]. Moreover, it has been shown by Sterzing and Barton [10] that hydrogenation of mixtures of egg-yolk phosphatidylcholine and beef-brain phosphatidylserine decreases procoagulant activity, but this effect could be reversed upon increasing the degree of unsaturation or by addition of cholesterol. These investigators also demonstrated that the phase transition of beef-brain phosphatidylserine shifts from −14 °C to 72 °C upon hydrogenation, which led them to propose that restoration of procoagulant activity is related to the phase-transition temperature of the lipid mixtures. However, a direct relationship with the lipid phase transition can only be established when synthetic phospholipids with well-defined fatty acids are used, having a transition temperature in the range where clotting assays can be performed.

In the present study, the clot-promoting behaviour in a one-stage prothrombinase assay of synthetic mixtures of phosphatidylserine and phosphatidylcholine is compared with their phase transition and mixing properties as observed calorimetrically. The results demonstrate that melting of the lipids is indeed accompanied with a sharp increase in coagulant activ-

Abbreviation. Activation enzyme RVV-X, factor-X-activating enzyme from Russell's viper venom.

ity, while phase separations of the lipids have a profound influence on the lipid concentration required for maximal activity.

MATERIALS AND METHODS

Lipids

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (dioleoylphosphatidylcholine), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (dipalmitoylphosphatidylcholine), and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (dimyristoylphosphatidylcholine) were prepared by reacylating the cadmium chloride adduct of *sn*-glycero-3-phosphocholine with the appropriate fatty acyl chloride according to the method described by Baer and Buchnea [11]. Dry ether was used instead of dry chloroform to achieve higher yields (H. M. Verhey, personal communication). 1,2-Dioleoyl-*sn*-glycero-3-phosphoserine (dioleoylphosphatidylserine), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoserine (dipalmitoylphosphatidylserine and 1,2-dimyristoyl-*sn*-glycero-3-phosphoserine (dimyristoylphosphatidylserine) were prepared from the respective phosphatidylcholines by enzymatic synthesis as described by Comfurius and Zwaal [12]. Cholesterol (99% pure) was obtained from Merck. All other reagents were of analytical grade.

Coagulation Assay

0.1 ml delipidated bovine plasma (diluted five-fold with Michaelis buffer pH 7.4) was incubated for 30 s with 0.1 ml activation enzyme RVV-X (factor-X-activating enzyme from Russell's viper venom) solution in Michaelis buffer (1 µg protein/ml) and 0.1 ml $CaCl_2$ (15 mM) to achieve activation of factor X [13]. 0.1 ml phospholipid vesicle suspension was added in concentrations ranging from 1000-1 nmol/ml and the clotting time was measured. The concentration of activation enzyme RVV-X was used as adjustable parameter to set the coagulation time at about 225 s when Michaelis buffer was added instead of lipid vesicles.

Delipidated bovine plasma was obtained from normal bovine citrated plasma adjusted to a density of 1.21 g/ml with KBr, using a flotation density technique [14]. Centrifugation was carried out at $350000 \times g$ for 36 h at 4 °C. The resulting delipidated plasma was dialyzed against a solution of 150 mM NaCl and 10 mM sodium citrate. Alternatively, plasma was delipidated with butanol/di-isopropyl ether (40/60, v/v) using the method described by Cham and Knowles [15]. Both methods gave similar results. Factor V was determined by a one-stage assay using factor-V-deficient reagent [16]. About 50% of the normal value was present in both delipidated plasmas. Factor II and factor X were also determined in a one-stage assay using the respective factor-deficient reagents [17,18]. These factors were present in concentrations ranging from 80 – 100% of the normal values. Activation enzyme RVV-X, purified according to the method of Schiffman et al. [19], was a gift of Mrs. Govers-Riemslag. No phospholipase A activity could be detected in this preparation. The lipid vesicle solutions were prepared by drying 5 µmol of lipid under nitrogen followed by sonication for 10 min above the phase transition temperature in 5 ml Michaelis buffer using a MSE ultrasonic disintegrator (mark II 150 W) set at 10 µm peak to peak amplitude. After sonication no pH adjustment was needed.

Differential Scanning Calorimetry

Differential scanning calorimetry was used to detect the phase transitions and also to investigate the miscibility properties of the mixtures of the lipids used. 5 µmol lipid was dried in the sample pan under nitrogen and drying was completed in vacuum for at least three hours [20]. After addition of 15 µl buffer the sample pan was sealed. Before the final scans were made the samples were scanned through the phase transition at least five times and then incubated for ten minutes above the phase transition. Michaelis buffer/ethylene glycol (1/1, v/v) was used in samples containing dioleoylphosphatidylcholine or dioleoylphosphatidylserine. In all other samples only Michaelis buffer was used. Scans were made on a Perkin-Elmer DSC-1 B calorimeter at a rate of 8 °C/min using heating scans only. For the construction of the phase diagram of the dimyristoylphosphatidylserine/dipalmitoylphosphatidylcholine binary mixture, three heating and three cooling curves were recorded at a rate of 4 °C/min. The diagram was constructed according to the method described by Van Dijck et al. [20].

RESULTS

The calorimetric behaviour of a number of mixtures of synthetic phosphatidylserines and phosphatidylcholines was compared with their clot-promoting activity in the one-stage prothrombinase assay.

Dioleoylphosphatidylserine/ Dioleoylphosphatidylcholine

These mixtures were investigated because the phospholipid bilayers are in the liquid crystalline phase, well above the phase transition, at physiological temperature. The calorimetric data indicate complete miscibility for all compositions as is to be expected from the almost identical transition temperature for

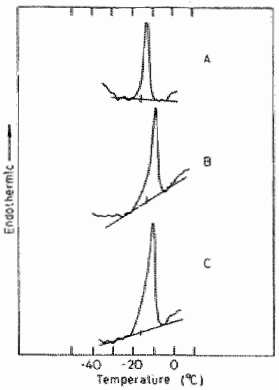


Fig. 1. *Differential scanning calorimetric heating scans of dioleoylphosphatidylserine (A), dioleoylphosphatidylcholine (C) and a 1/1 (mol/mol) mixture (B).* Scans were recorded at a rate of 8 °C/min. Transition temperatures are obtained as the intersection of the slope of the ascending arm with the baseline. The transition temperatures are −15.5 °C for PS, −16 °C for PC and −14.5 °C for the mixture

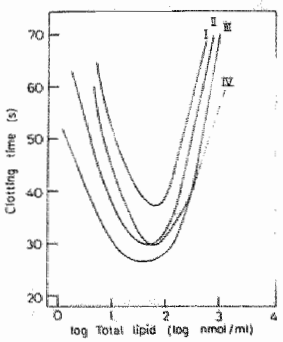


Fig. 2. *Clotting times for different compositions of dioleoylphosphatidylserine/dioleoylphosphatidylcholine mixtures as a function of the logarithm of the total lipid concentration expressed in nmol of added lipid.* For reasons of clarity the experimental points are omitted and only the fitted curves shown. The different curves represent different mole fractions of phosphatidylserine (I) 100; (II) 80; (III) 50; (IV) 20 mol/100 mol

the phosphatidylserine and the phosphatidylcholine (−15.5 °C and −16 °C, respectively). The transition temperature for the pure phosphatidylcholine is slightly higher than found by De Kruyff et al. [21], who measured in water/ethylene glycol (1/1, v/v). They found the transition at −20 °C. Fig. 1 shows the heating curves for the single compounds and the 50/50 (mol/mol) mixture. All mixtures tested (80/20, 60/40, 50/50, 40/60, 20/80, mol/mol) had their solidus point at about −14.5 °C. Under the present conditions it was not feasible to construct a phase diagram as in our buffer system the lipids showed considerable hysteresis.

When investigating the coagulant activity of the phosphatidylserine and the mixtures, in all cases the maximal procoagulant activity was found at the same phospholipid concentration (Fig. 2). Starting at concentrations of 1 nmol/ml of lipid added, the clotting time became shorter until a minimum was reached at about 75 nmol/ml added lipid. At higher lipid concentrations a gradual increase of the clotting time was observed. Mixtures ranging from 40–70 mol of phosphatidylserine/100 mol showed maximal procoagulant activity (about 27 s clotting time) while mixtures containing 20 or 80 mol of phosphatidylserine/100 mol were slightly less active (about 31 s). The maximal activity for the pure phosphatidylserine alone was found to be considerably smaller (about 40 s clotting time) confirming that lipid mixtures form a better catalytic surface in the prothrombinase complex than the single compound. Phosphatidylcholine alone showed almost no activity since coagulation times in the order of the buffer time were observed.

Dipalmitoylphosphatidylserine/ Dipalmitoylphosphatidylcholine

These mixtures were studied because, in contrast to the mixtures described above, both phospholipids are in the gel phase at physiological temperature. In agreement with the literature [22, 23], the transition temperatures for the pure phosphatidylserine and phosphatidylcholine were found to be 57 °C and 41.5 °C, respectively. The calorimetric data of the mixtures indicated solid/solid immiscibility because they did not give a single transition peak (results not shown). In the coagulation assay these mixtures also showed maximal procoagulant activity at defined phospholipid concentrations but in contrast to the dioleoyl mixtures described above, the concentrations were found to be significantly higher. Moreover, the clotting times were found to be considerably longer (Fig. 3). For the most active mixture (50 mol of phosphatidylserine/100 mol) a minimal clotting time of 58 s was measured.

It has been shown that introducing cholesterol in bilayers of phosphatidylcholine has a condensing effect on bilayers in the liquid crystalline phase and a liquefying effect on bilayers in the gel state [24]. This intermediate state appears to be almost liquid crystalline. The lateral diffusion rate of the lipid molecules in the bilayer in the liquid crystalline phase is reduced

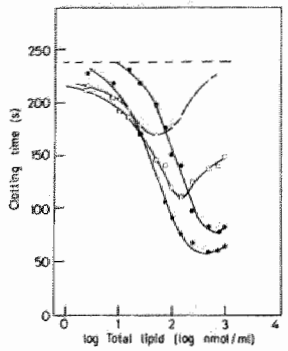


Fig. 3. *Clotting times for different compositions of dipalmitoylphosphatidylserine/dipalmitoylphosphatidylcholine mixtures as a function of the logarithm of the total lipid concentration expressed in nmol/ml of added lipid.* The different curves represent different mole fractions of phosphatidylserine: (O—O) 100; (□—□) 80; (✱—✱) 50; (●—●) 20 mol/100 mol. The dashed line represents the pure phosphatidylcholine being identical to the buffer value

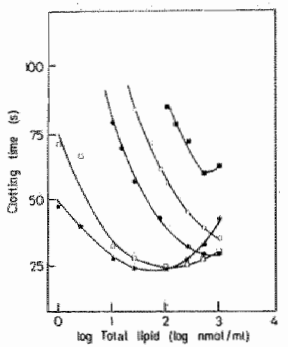


Fig. 4. *Clotting times of the 50:50 (mol/mol) mixture of dipalmitoylphosphatidylserine and dipalmitoylphosphatidylcholine with different amounts of cholesterol.* (■—■) No cholesterol; (O—O) 15; (●—●) 20; (□—□) 33 mol/100 mol. As a control the 50:50 (mol/mol) mixture of dioleoylphosphatidylserine and dioleoylphosphatidylcholine with 33 mol cholesterol/100 mol was also measured (✱—✱)

two-fold upon mixing with cholesterol, whereas it goes down 10-times when going from the liquid crystalline to the gel phase [25]. The effect was tested by introducing cholesterol up to amounts of 33 mol/100 mol total lipid since at this ratio the effect on the phase transition is completed [24]. The addition of cholesterol to a 50/50 (mol/mol) mixture of the dipalmitoyl compounds had a dramatic effect on the procoagulant activity.

Although addition of cholesterol to the dioleoyl mixture shows somewhat shorter clotting times (compare Fig. 2) it is difficult to assess whether this is significant since variations of approximately 5% may occur from day to day. From Fig. 4 it is clear that the addition of cholesterol up to 33 mol/100 mol to a phospholipid bilayer in the gel state reduces the coagulation time to become almost equal to that of a mixture in the liquid crystalline state. Moreover, the lipid concentration at which maximal procoagulant activity was observed, shifted to lower values.

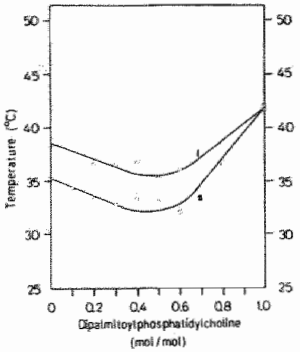


Fig. 5. *Phase diagram of the dimyristoylphosphatidylserine/dipalmitoylphosphatidylcholine binary mixture constructed from calorimetric data.* Scans were made at a rate of 4 °C/min. The points on the solidus (s) were taken as an average of three scans and were constructed as the intercept of the slope of the ascending arm of the heating curve with the baseline. Points on the liquidus(l) were taken as the average of three cooling curves and constructed as the intercept of the slope of the descending arm of the cooling curve with the baseline. As the mixing behaviour is non-ideal it seemed not feasible to construct a theoretical diagram. The solid line represents a fit to the experimental points

Dimyristoylphosphatidylserine/ Dipalmitoylphosphatidylcholine

In order to establish a direct correlation between melting of the fatty acyl chains and coagulant activity, it was decided to investigate the mixture of dimyristoylphosphatidylserine and dipalmitoylphosphatidylcholine because both lipids have their phase transition not far from physiological temperature. The phase diagram constructed from calorimetric data is shown in Fig. 5. In agreement with other investigators, the transition temperature determined from the heating curves for the phosphatidylserine and the phosphatidylcholine were found to be 35.0 °C and 41.5 °C respectively [12, 23]. It was observed that the two compounds exhibit miscibility in both phases but the mixing behaviour is non-ideal. The coagulant activity of mixtures of these two lipids showed marked similarity to the behaviour of the two dioleoyl compounds (Fig. 6). For 100, 80, 60, 50, and 40 mol of phosphati-

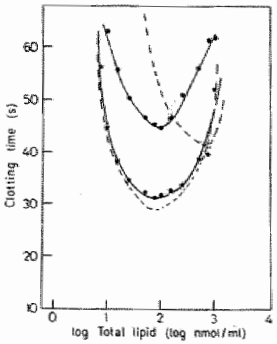


Fig. 6. *Clotting times for various compositions of dimyristoylphosphatidylserine/dipalmitoylphosphatidylcholine mixtures as a function of the logarithm of the total lipid concentration expressed in nmol/ml of added lipid.* The different curves represent different mole fractions of phosphatidylserine: (●——●) 100; (∗ ---∗) 80; (○----○) 50; (□----□) 20 mol/100 mol

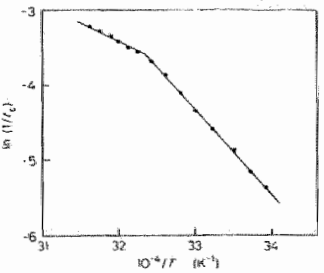


Fig. 8. *Arrhenius plot of the clotting times obtained with an equimolar mixture of dimyristoylphosphatidylserine and dipalmitoylphosphatidylcholine at a concentration of 100 nmol/ml of added lipid*

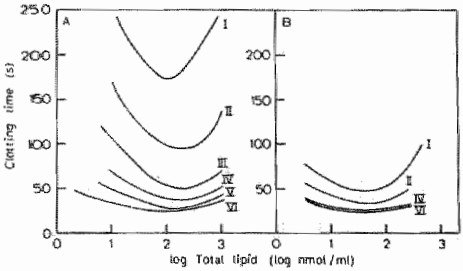


Fig. 7. *Temperature-dependent clotting times for the 50/50 (mol/mol) mixtures of (A) dimyristoylphosphatidylserine and dipalmitoylphosphatidylcholine and (B) dioleoylphosphatidylserine and dioleoylphosphatidylcholine as a function of the logarithm of total lipid concentration expressed in nmol/ml of added lipid.* The different curves represent different temperatures: (I) 25 °C; (II) 30 °C; (III) 33 °C; (IV) 37 °C; (V) 39 °C; (VI) 42 °C

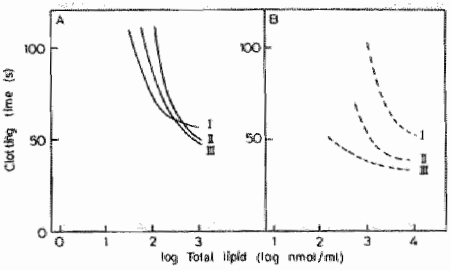


Fig. 9. *Temperature-dependent clotting times for (A) vesicle and (B) liposomes of the 20/80 (mol/mol) mixture of dimyristoylphosphatidylserine and dipalmitoylphosphatidylcholine as a function of total lipid concentration expressed in nmol/ml of added lipid.* The different curves represent different temperatures. (A): (I) 35 °C; (II) 40 °C; (III) 43 °C. (B): (I) 35 °C; (II) 39 °C; (III) 42 °C

dylserine/100 mol there is excellent agreement with the phase diagram which predicts liquid crystalline behaviour at physiological temperature. Since the phase diagram predicts that a 50/50 mol/mol mixture will be in the gel state below 33 °C the procoagulant activity of this mixture was compared with a mixture of the dioleoyl compounds at temperatures between 25 °C and 43 °C. The difference between the two mixtures vanished almost completely upon going through the phase transition (Fig. 7). As it has been shown that the clotting time is inversely proportional to the velocity of coagulation [37] an Arrhenius plot of the data obtained with the optimal lipid concentration (100 nmol/ml added lipid) was constructed. This showed a sharp increase in coagulant activity at 0.00324 K^{-1} (35.9 °C) which corresponds closely to the phase transition temperature of the lipid mixture (Fig. 8).

The behaviour of the 20/80 (mol/mol) mixture containing 20 mol of phosphatidylserine/100 mol proved to be of special interest. As expected from the phase diagram, this mixture is in the gel phase at physiological temperature, which may be responsible for its weaker coagulant activity. However, no sharp increase in coagulant activity was observed when this mixture was heated above its phase transition temperature (Fig. 9 A). On the other hand, the expected jump in coagulation time did occur indeed when hand-shaken liposomes were used (Fig. 9 B). Since less lipid is available at the surface of liposomes as compared to vesicles [26], the optimal lipid concentrations shift to higher values.

Dimyristoylphosphatidylserine/ Dimyristoylphosphatidylcholine

Although the optimal lipid concentration where maximal procoagulant activity was found is the same with lipid mixtures in the liquid crystalline state (50 – 100 nmol/ml added lipid), a marked difference was observed between lipids in the gel state. The mixture of the dipalmitoyl compounds exhibited maximal procoagulant activity at 500 – 750 nmol/ml added lipid, whereas the equimolar mixture of dimyristoylphosphatidylserine and dipalmitoylphosphatidylcholine had its optimum at about 100 nmol/ml added lipid both below and above the phase transition. Therefore, a mixture of the two dimyristoyl compounds was investigated since the coagulant behaviour of this mixture could also be measured above and below the phase transition.

The miscibility properties of dimyristoylphosphatidylserine and dimyristoylphosphatidylcholine were found to be similar to the mixtures of the dipalmitoyl compounds. Melting started at the transition temperature of the dimyristoylphosphatidylcholine (23 °C) and was completed at about 30 °C, indicating solid/solid immiscibility. When the coagulant behaviour of an equimolar mixture was tested as a function of temperature, a sharp increase in the coagulant activity was observed on going through the phase transition (Fig. 10). However, in contrast to the mixture of dimyristoylphosphatidylserine and dimyristoylphosphatidylcholine, the optimal lipid concentration for maximal procoagulant activity shifted from about 500 nmol/ml added lipid at 23 °C to 50 – 100 nmol/ml added lipid at 37 °C.

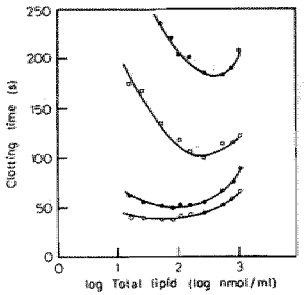


Fig. 10. *Temperature-dependent clotting times for the equimolar mixture of dimyristoylphosphatidylserine and dimyristoylphosphatidylcholine as a function of the logarithm of the lipid concentration expressed as nmol/ml of added lipid.* (■——■) 23 °C; (□——□) 27 °C; (●——●) 32 °C; (○——○) 37 °C

DISCUSSION

The influence of phospholipids on the clotting time of plasma in the presence of activation enzyme RVV-X provides direct information of the lipid requirements of the prothrombinase complex. Since factor X is activated by incubation with the activation enzyme RVV-X and $CaCl_2$ prior to addition of lipids, no contributions of the accelerating effect of phospholipids on the intrinsic and extrinsic factor X activator are to be expected [5].

The results show excellent correlation between liquidity of the phospholipid bilayer as detected with differential scanning calorimetry and the procoagulant activity measured in the coagulation assay. The minimal clotting times for phospholipids in the liquid crystalline phase are significantly shorter than those observed for lipids in the gel phase. For example, equimolar mixtures of dioleoylphosphatidylserine and dioleoylphosphatidylcholine (liquid crystalline) at about 2% of their optimal concentration exhibit clotting times of 60 s which are comparable with the minimal clotting times of equimolar mixtures of dipalmitoylphosphatidylserine and dipalmitoylphosphatidylcholine. Assuming that the prothrombinase complex will be equally active with a solid and a liquid crystalline lipid surface, it can be tentatively concluded that the maximal number of active prothrombinase complexes formed in the presence of phospholipids in the gel phase is some 50-times smaller than with phospholipids in the liquid crystalline phase, provided that prothrombin is in excess in both cases. It can be ruled out that the observed lower coagulant activity of lipids in the gel phase is caused by a minor contamination of lower melting lipids, because addition of the dioleoyl mixture to the dipalmitoyl mixture in a ratio of 2 mol/100 mol prior to sonication has no significant influence on the coagulant behaviour.

The relation between acyl chain fluidity of the phospholipids and their procoagulant activity is further supported by the experiments using cholesterol. The disappearance of the difference between the 50/50 (mol/mol) mixture of the dipalmitoyl compounds and the 50/50 (mol/mol) mixture of the dioleoyl compounds upon introducing cholesterol into the bilayer is consistent with the observations of Sterzing and Barton [10]. These authors carried out experiments with bovine brain phosphatidylserine and with mixtures of this lipid with egg-yolk lecithin. From the increase in coagulant activity upon introducing cholesterol into bilayers of hydrogenated phospholipids and from the increase in activity when the degree of unsaturation of the acyl chains was increased, these authors concluded that the restoration of activity was related to the transition temperatures of the lipid mixtures. It should be mentioned, however, that this was not demonstrated in a direct experiment showing that the lipids acquire procoagulant activity when scanned through the phase transition temperature.

Direct evidence is provided by the results obtained with the mixture of dimyristoylphosphatidylserine and dipalmitoylphosphatidylcholine, showing a striking correlation between the coagulant activity and the phase diagram. Furthermore, an Arrhenius plot for the 50/50 (mol/mol) mixture indicates a sharp increase in coagulant activity when going through the phase transition, thus allowing the conclusion that melting of the fatty acyl chains (and not for example a pretransition of phosphatidylcholine occurring some 10 C below the actual phase transition) is indeed the determining parameter.

The apparent exception formed by the 20/80 (mol/mol) dimyristoylphosphatidylserine/dipalmitoylphosphatidylcholine mixture may be of further interest. It has been shown that with vesicles, phosphatidylserine has a preference for the inner half of the bilayer [27]. It can be expected that the asymmetric distribution is enhanced when the hydrocarbon chains of phosphatidylserine are shorter than the chains of phosphatidylcholine, as is the case with these mixtures. Therefore, it is reasonable to suppose that in this mixture the amount of phosphatidylserine at the outer surface will be lower than the critical value of phosphatidylserine required to show procoagulant activity, which is presumably not the case when liposomes of the same composition are used. In the other mixtures this effect was only observed when the phosphatidylserine content became less than 10 mol/100 mol which is in agreement with the findings of Bull and Barton [28]. Also, the lipid concentration at which the maximal procoagulant activity is reached shifts to values about eight times higher when liposomes are used instead of vesicles, which is in agreement with the observation that the available surface diminishes about eight-fold in liposome suspensions [26].

In order to be active in coagulation, the phospholipid/water interface must provide binding sites for the vitamin-K-dependent clotting factors X_a and II, as well as for factor V. It has been suggested by Subbaiah et al. [29] that a negative lipid surface is also required to bind factor V. Preliminary investigations carried out in our laboratory by Dr Rosing show that factor V fails to bind to pure egg-yolk lecithin whereas binding is observed when brain phosphatidylserine is present in the bilayer. Moreover, it was found that the binding decreases about five-fold when the phospholipids are in the gel phase thus supporting the notion that the interaction with factor V is also hydrophobic. On the other hand, the interactions with the vitamin-K-dependent clotting factors are thought to be sheerly electrostatic. These clotting factors presumably bind to negatively charged lipids through Ca ion bridging. In agreement with the literature, it was found that the bilayer must have a net negative surface charge [6–8]. However, in contrast to the general view the magnitude of this charge can vary considerably without influencing the coagulant behaviour. Although a negative charge seems to be required, the amount of available lipid surface as well as the physical state of the lipids appears to be more critical.

The appearance of an optimal lipid concentration is thought to result from the balancing effect of the density of bound proteins on the phospholipid surface and the lateral diffusion rates of these proteins over the surface. At low concentrations the density will be maximal. The more phospholipid surface is provided the more prothrombinase complexes will be formed and the clotting time becomes shorter. However, at higher concentrations the density of the bound proteins will decrease and their collision chance will therefore decrease also. This effect tends to prolong the clotting times. Thus, the concentration at which maximal activity is reached is expected to be a measure of the amount of active phospholipid surface involved as well as of the number of prothrombinase complexes formed. This is of course also influenced by the concentrations of the clotting factors in the plasma. The binary mixtures which show complete miscibility in both phases (the dioleoyl mixtures and the mixtures of dimyristoylphosphatidylserine and dipalmitoylphosphatidylcholine) have an optimal concentration of about 100 nmol/ml added lipid. The dipalmitoylphosphatidylserine also has an optimal concentration of about 100 nmol/ml added lipid whereas the binary mixtures of dipalmitoylphosphatidylserine and dipalmitoylphosphatidylcholine as well as the mixtures of the two dimyristoyl compounds at temperatures below their phase transitions have optimal concentrations at significantly higher values. These latter mixtures show only partial miscibility in the solid state because the calorimetric heating scans show two peaks of which one can be identified as the peak of the phosphatidylcholine. The second peak is presumably produced by the transition of a mixture of both lipids, since the transition temperature lies between those of the pure substances. Since phosphatidylcholine alone has no procoagulant activity it can be assumed that only part of the available lipid surface of mixtures showing phase separation contributes to the formation of prothrombinase. This may explain why higher amounts of lipids are needed to reach maximal coagulant activity with these mixtures. Assuming that with the two dimyristoyl compounds, complete mixing occurs above the phase transition, it may be concluded that the shift in optimal concentration which is observed on measuring coagulant activity between 23 and 37 °C is a reflection of the amount of mixed surface. Since no shift occurs with the mixture of dimyristoylphosphatidylserine and dipalmitoylphosphatidylcholine, it is unlikely that the total surface area available for catalysis is the determining parameter, also because all lipid vesicles have been prepared by sonication above the phase transition. Although

it is not ruled out that small variations of vesicle size among the various preparations may occur, it can be calculated from [38] that the amount of lipid present on vesicles with a diameter of 25 nm is about 65% while this value decreases to 57% when the vesicle size is doubled. This is not sufficient to explain the magnitude of the shift described above.

It is generally known that Ca^{2+} is an essential component of the prothrombinase complex. It is also known that Ca^{2+} can have very drastic effects on the physical state of a phospholipid bilayer [30–32]. In mixtures of phosphatidylserine and phosphatidylcholine, Ca^{2+} can induce a phase separation of the two phospholipids [33], which is thought to be caused by the clustering of the serine head groups bridged together by the Ca ions thus 'freezing out' the phosphatidylserine from the liquid crystalline phosphatidylcholine matrix. These phase separations are expected to occur rapidly [34, 35]. However, the results presented above do not show a correlation between coagulant activity and the physical state of the bilayers when the effect of Ca ions is taken into account. This is markedly illustrated by a control experiment using 50/50 (mol/mol) mixture of dimyristoylphosphatidylserine and dipalmitoylphosphatidylcholine. Prior to addition in the coagulation assay, the vesicles were incubated with varying amounts of $CaCl_2$ up to a ratio Ca^{2+}/phosphatidylserine of 20 : 1. Even when these incubations were carried out for several hours, no change in coagulation behaviour could be detected. At present, it is not clear why a perfect correlation is observed between the physical state of the bilayers before addition to the coagulation assay and not to the expected state when Ca ions are present. Recently, Barton and Findlay [36] suggested that prothrombin activation occurs most efficiently at the lateral interface between the gel state of an acidic phospholipid-Ca^{2+} complex (e.g. phosphatidylglycerol) and the liquid crystalline state of a neutral phospholipid (e.g. phosphatidylcholine). However, this explanation seems hardly applicable here as coagulation times are only slightly influenced by the phosphatidylserine/phosphatidylcholine ratio, which should strongly influence the amount of lateral interface. Moreover, it appears possible to correlate the lipid concentrations at which maximal coagulant activity is reached with the miscibility properties of the mixtures, which indicates that caution should be exercised when only one lipid concentration is used in a coagulation assay.

One of us (H. v. Zutphen) was supported by the Foundation for Medical Research FUNGO which is subsidized by the Netherlands Organisation for the Advancement of Pure Research (Z.W.O.). Part of the phospholipids were synthesized at the Gaubius Institute, Health Research Organization T.N.O. at Leiden (The Netherlands) with the assistance of Ms L. M. van Hevelingen.

REFERENCES

1. Marcus, A. J. (1966) *Adv. Lipid Res. 4*, 1–37.
2. Jackson, C. M., Owen, W. G., Gitel, S. N. & Esmon, C. T. (1977) *Thromb. Diath. Haemorrh. 57 (Suppl.)* 273–293.
3. Hemker, H. C. (1975) in *Handbook of Hemophilia* (Brinkhous, K. M. & Hemker, H. C., eds) pp. 31–48, Excerpta Medica, Amsterdam.
4. Zwaal, R. F. A. (1978) *Biochim. Biophys. Acta, 515*, 163–205.
5. Suttie, J. W. & Jackson, C. M. (1977) *Physiol. Rev. 57*, 1–70.
6. Bangham, A. D. (1961) *Nature (Lond.) 192*, 1197–1198.
7. Papahadjopoulos, D. P., Hougie, C. & Hanahan, D. J. (1962) *Proc. Soc. Exp. Biol. Med. 111*, 412–416.
8. Daemen, F. J. M., van Arkel, C., Hart, H. C., van der Drift, C. & van Deenen, L. L. M. (1965) *Thromb. Diath. Haemorrh. 13*, 194–217.
9. Grisdale, P. J. & Okary, A. (1965) *Can. J. Biochem. 13*, 1465–1470.
10. Sterzing, P. R. & Barton, P. G. (1973) *Chem. Phys. Lipids, 10*, 137–148.
11. Baer, E. & Buchnea, D. (1959) *Can. J. Biochem. Physiol. 37*, 953–959.
12. Comfurius, P. & Zwaal, R. F. A. (1977) *Biochim. Biophys. Acta, 488*, 36–42.
13. Lindhout, M. J., Kop-Klaassen, B. H. M. & Hemker, H. C. (1978) *Biochim. Biophys. Acta, 533*, 342–354.
14. Havel, R. J., Eder, H. A. & Bragdon, J. H. (1955) *J. Clin. Invest. 34*, 1345–1351.
15. Cham, B. E. & Knowles, B. R. (1976) *J. Lipid Res. 17*, 176–181.
16. Borchgrevink, C. F., Pool, J. G. & Stormorken, H. (1960) *J. Lab. Clin. Med. 55*, 625–633.
17. Vermeer, C., Soute, B. A. M. & Hemker, H. C. (1977) *Thromb. Res. 10*, 495–507.
18. Vermeer, C., Soute, B. A. M., Ates, G. & Brummelhuis, H. G. J. (1976) *Vox. Sang. 30*, 1–22.
19. Schiffman, S., Theodor, I. & Rapaport, S. I. (1969) *Biochemistry, 8*, 1397–1405.
20. Van Dijck, P. W. M., Kaper, A. J., Oonk, H. A. J. & de Gier, J. (1977) *Biochim. Biophys. Acta, 470*, 58–69.
21. De Kruyff, B., Demel, R. A., Slotboom, A. J., van Deenen, L. L. M. & Rosenthal, A. F. (1973) *Biochim. Biophys. Acta, 307*, 1–19.
22. Mc Donald, R. C., Simon, S. A. & Baer, E. (1976) *Biochemistry, 15*, 885–891.
23. Ladbrooke, B. D. & Chapman, D. (1969) *Chem. Phys. Lipids, 3*, 304–367.
24. Demel, R. A. & de Kruyff, B. (1976) *Biochim. Biophys. Acta, 457*, 109–132.
25. Cullis, P. R. (1976) *FEBS Lett. 70*, 223–228.
26. Bangham, A. D., de Gier, J. & Greville, G. D. (1967) *Chem. Phys. Lipids, 1*, 225–246.
27. Berden, J. A., Barker, R. W. & Radda, G. K. (1975) *Biochim. Biophys. Acta, 375*, 186–208.
28. Bull, R. K., Jevons, S. & Barton, P. G. (1972) *J. Biol. Chem. 247*, 2747–2754.
29. Subbaiah, P. V., Bajwa, S. S., Smith, C. M. & Hanahan, D. J. (1976) *Biochim. Biophys. Acta, 444*, 131–146.
30. Tocanne, J. F., Ververgaert, P. H. J. Th., Verkleij, A. J. & van Deenen, L. L. M. (1974) *Chem. Phys. Lipids, 12*, 201–219.
31. Van Dijck, P. W. M., Ververgaert, P. H. J. Th., Verkleij, A. J., van Deenen, L. L. M. & de Gier, J. (1975) *Biochim. Biophys. Acta, 406*, 465–478.
32. Papahadjopoulos, D., Vail, W. J., Pangborn, W. A. & Poste, G. (1976) *Biochim. Biophys. Acta, 448*, 265–283.
33. Papahadjopoulos, D., Vail, W. J., Newton, C., Nir, S., Jacobson, K., Poste, G. & Lazo, R. (1977) *Biochim. Biophys. Acta, 465*, 579–598.

34. Ohnishi, S. & Ito, T. (1974) *Biochemistry*, *13*, 881–887.
35. Ito, T. & Ohnishi, S. (1974) *Biochim. Biophys. Acta*, *352*, 29–37.
36. Barton, P. G. & Findlay, E. J. (1977) in *The Significance of Platelet Function Tests in the Evaluation of Hemostatic and Thrombosis Tendencies* (Day, H. J., Zucker, M. B. & Holmsen, H., eds) pp. 462–470, U.S. Natl. Inst. Health, Bethesda, Md.
37. Hemker, H. C., Kop, J. & Willems, G. M. (1978) *Thromb. Haemostasis* in the press.
38. De Kruijff, B., Cullis, P. R. & Radda, G. K. (1975) *Biochim. Biophys. Acta*, *406*, 6–20.

G. Tans, H. van Zutphen, P. Comfurius, H. C. Hemker, and R. F. A. Zwaal*,
Capaciteitsgroep Biochemie, Biomedisch Centrum, Rijksuniversiteit Limburg,
Postbus 616, NL-6200 MD Maastricht, The Netherlands

* To whom correspondence should be addressed.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 255, No. 1, Issue of January 10, pp. 274-283, 1980
Printed in U.S.A.

The Role of Phospholipids and Factor V_a in the Prothrombinase Complex*

(Received for publication, December 5, 1978, and in revised form, May 17, 1979)

Jan Rosing, Guido Tans, José W. P. Govers-Riemslag, Robert F. A. Zwaal, and H. Coenraad Hemker
From the Department of Biochemistry, Biomedical Centre, State University of Limburg, Maastricht, The Netherlands

The kinetic parameters of the conversion of bovine prothrombin into thrombin by activated bovine blood clotting factor X (X_a) have been determined in the absence and presence of Ca^{2+}, activated bovine factor V (V_a), and phospholipid (dioleoylphosphatidylcholine/dioleoylphosphatidylserine, 1:1; mol/mol). In the absence of accessory components, the K_m for prothrombin is 131 μM, which is well above its concentration in bovine plasma of about 1.5 μM. The V_{max} of thrombin formation is 0.61 mol min^{-1} mol of X_a^{-1} under these conditions. In the presence of 7.5 μM phospholipid, the K_m drops to 0.058 μM and the V_{max} slightly increases to 2.25 mol min^{-1} mol of X_a. For the complete prothrombinase complex (X_a, V_a, Ca^{2+}, and 7.5 μM phospholipid), a K_m for prothrombin of 0.21 μM and a V_{max} of 1919 mol min^{-1} mol of X_a^{-1} is found. The V_{max} of thrombin formation slightly increases when more phospholipid is present in our experiments and there is a considerable increase of the K_m for prothrombin at higher phospholipid concentrations. Preliminary calculations show that the prothrombin density at the phospholipid surface at the K_m is independent of the phospholipid concentration. This indicates that the K_m measured in the presence of phospholipid has to be regarded as an apparent K_m and the local prothrombin concentration determines the kinetics of activation.

Prothrombin activation by prothrombinase complexes of different compositions was followed by gel electrophoresis in the presence of sodium dodecyl sulfate. Both in the absence and presence of phospholipid but without factor V_a, prethrombin 2 is the main product formed during the initial stages of steady state prothrombin activation. In the presence of factor V_a, thrombin is the main end product and minute amounts of prethrombin 2 are formed. This shift in the reaction pathway of prothrombin activation caused by factor V_a will contribute to the observed increase of the V_{max} measured in the presence of factor V_a.

One of the key reactions in blood coagulation and hemostasis is the formation of thrombin by limited proteolysis of its zymogen, prothrombin.[1] Several proteolytic enzymes can bring about this reaction, but under physiological conditions the serine protease factor X_a is the activating enzyme. In the last 25 years many papers have appeared in the literature describing the detection and isolation of partial proteolysis products of prothrombin (see Ref. 1). They finally resulted in a series of papers by the group of Jackson *et al.* (2-7) which completed the description of the sites of peptide-bond splitting and order of bond cleavage during prothrombin activation. Papahadjopoulos and Hanahan (8) as well as several other authors (see Ref. 1) have shown that additional components are essential for prothrombin activation during *in vitro* blood coagulation. It is now generally accepted that Ca^{2+}, a phospholipid surface, and factor V_a are required for prothrombin activation under physiological conditions. It has been shown that the above mentioned clotting factors have to be absorbed on the phospholipid bilayer surface in order to acquire efficient interaction. Both prothrombin and factor X_a bind to the phospholipid surface via calcium bridges between γ-carboxyglutamic acids present in these proteins and polar head groups of the phospholipid molecules (9, 10), while hydrophobic interactions play an important role in the binding of factor V_a (11). For a recent review about phospholipid involvement in blood coagulation, see Ref. 12.

Not much is known, however, about the mechanism responsible for the enhancement of the rate of prothrombin activation by factor V_a and phospholipids. Esmon and Jackson (5) did not find evidence for a change of sites and orders of bond cleavage when the prothrombin molecule is activated by different combinations of the components of the prothrombinase complex. The enhancement of the rate of thrombin formation when phospholipid or factor V_a, or both, are added to a mixture of prothrombin, factor X_a, and $CaCl_2$ as such (7, 13), does not allow conclusions about the way they are involved in the enzymatic mechanism of prothrombin activation, since these experiments were carried out at single concentrations of the components of the prothrombinase complex.

The purpose of the experiments described in this paper was to assess the kinetic parameters (K_m for prothrombin and V_{max} of thrombin formation) for different prothrombin activating mixtures (*i.e.* factor X_a either in the absence or presence of Ca^{2+}, factor V_a, and/or phospholipid). This enables a precise quantitation of the observed rate enhancements and allows a first attempt to explain the role of the accessory components in the mechanism of prothrombin activation.

Silverberg *et al.* (14) have reported an example of the effect of an accessory component on the kinetics of a proteinase-catalyzed activation of a clotting factor. They studied the effect of tissue factor on the activation of factor X by factor VII_a. Tissue factor, a preparation which contains both phospholipids and protein components decreased the K_m for factor X about 10-fold and increased the K_{cat} 3000-fold. Our kinetic approach allows a separate assessment of the effects of phospholipid and the protein accessory component (factor V_a) of

* This investigation was partially supported by the Netherlands Organization for the Advancement of Pure Scientific Research (Z.W.O.). Part of the chromogenic substrates used in our laboratory were kindly donated by AB Kabi Diagnostica. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The nomenclature of the blood coagulation factors used is that recommended by the Task Force on Nomenclature of Blood Clotting Zymogens and Zymogen Intermediates.

the prothrombinase complex on the kinetic parameters of prothrombin activation. The data presented in this paper likely have implications for the role of phospholipid and factor VIII in the factor X-activating complex (factor IX_a, factor VIII, Ca^{2+}, and phospholipid) of the intrinsic pathway of blood coagulation.

EXPERIMENTAL PROCEDURES

Materials—S 2238[2] and S 2222 were purchased from AB Kabi Diagnostica, Stockholm, Sweden. *p*-NPGB was from Nutritional Biochemicals. Russell's viper venom, soybean trypsin inhibitor, and ovalbumin were obtained from Sigma. DEAE-Sephadex A-50, QAE-(quarternaryammoniumethyl) Sephadex A-50, SP (sulfopropyl) Sephadex C-50, Sephadex G-100, Sepharose 4B and 6B, and CNBr-activated Sepharose 4B were obtained from Pharmacia. QAE-Cellulose was a product of Schleicher and Schuell. *p*-Aminobenzamidine obtained from Merck was coupled to Sepharose 4B according to the procedure described by Di Scipio *et al.* (15). STI was coupled to CNBr-activated Sepharose 4B following the method of Cuatrecasas (16). All reagents used were of the highest grade commercially available.

Proteins—Bovine prothrombin was prepared according to the method of Owen *et al.* (2). Before storage at −80°C the prothrombin preparations were passed through a column (0.9 × 20 cm) of SP-Sephadex and STI-Sepharose 4B to reduce the small amounts of thrombin and factor X_a which might be present in these preparations. No thrombin and factor X_a could be detected in our final prothrombin preparations using an assay with the chromogenic substrates S 2238 and S 2222. Prothrombin concentrations were calculated from the E_{280} using $E^{1\%}_{280}$ = 15.5 (2) and 72,000 for the molecular weight of prothrombin (2). Bovine factors X_1 and X_2 were purified as described by Fujikawa *et al.* (17). Bovine factor X_a was prepared from factor X_2 using RVV-X according to the method of Fujikawa *et al.* (18). Factor X_a concentrations were calculated after active site titration according to Smith (19).

Prothrombin, factor X_1 and factor X_2 preparations were homogeneous and pure as determined by gel electrophoretic analysis in the presence of sodium dodecyl sulfate. The specific activities attained were equal to those reported in Ref. 2 (for prothrombin) and Ref. 17 (for factor X_1 and X_2).

RVV-X was purified from the crude venom by the method of Schiffman *et al.* (20). Factor V was purified according to the procedure of Smith and Hanahan (21) with minor modifications. The final preparation had a specific activity of 40 U/mg assuming 1 unit of factor V to be present per ml of normal bovine plasma. Factor V (0.4 mg/ml) was activated at 37°C for 15 min in 200 mM Tris-HCl, 50 mM NH_4Cl, 10% glycerol (pH 7.5) with thrombin (1.31 μg/ml). After this time interval the specific activity had risen to about 320 U/mg and did not increase upon longer incubation. Factor V_a was separated from thrombin on a benzamidine-Sepharose 4B column, which to our surprise also bound some of the factor V_a. The bound factor V_a could be eluted with 20 mM Tris, 200 mM NaCl (pH 7.5).

Thrombin was purified as a prothrombin activation product. Prothrombin (11.5 ml; 4.0 mg/ml) was activated with factor X_a (25 μg/ml) in a solution containing 20 mM Tris, 100 mM NaCl, 20 mM $CaCl_2$ (pH 7.5) at 37°C. After 20 min 3 ml of STI-Sepharose slurry was added to bind factor X_a. Ten minutes later this mixture was applied to QAE-Sephadex A-50 column (1.5 × 30 cm), and the prothrombin activation products were eluted with a linear gradient (2 × 150 ml) of 0.1 M to 0.6 M NaCl in 20 mM Tris at pH 7.5. The thrombin-containing fractions were applied to a benzamidine-agarose column (1.5 × 15 cm). Thrombin was eluted with a linear gradient (2 × 100 ml) of 0 to 0.3 M benzamidine in 0.1 M NaCl, 20 mM Tris at pH 7.5 (22). Benzamidine was removed by passing the thrombin-containing fractions through a Sephadex G-25 column (0.9 × 15 cm) followed by extensive dialysis against the same buffer without benzamidine at 5°C. The thrombin concentration was determined by active site titration with *p*-NPGB according to Chase and Shaw (23).

Phospholipids and Phospholipid Vesicle Preparations—1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (18:1$_{\omega 9}$/18:1$_{\omega 9}$-phosphatidylcholine) was prepared by reacylating the cadmium chloride adduct of *sn*-glycero-3-phosphocholine with the appropriate fatty acyl chloride according the method of Bear and Buchnea (24). 1,2-Dioleoyl-*sn*-glycero-3-phosphoserine (18:1$_{\omega 9}$/18:1$_{\omega 9}$-phosphatidylserine) was prepared from the respective phosphatidylcholine by enzymatic synthesis as described by Comfurius and Zwaal (25). Single bilayer vesicle solutions were prepared according to the method described by De Kruijff *et al.* (26) by sonication for 10 min in 20 mM Tris, 100 mM NaCl buffer at pH 7.5 at 0°C (above the phase transition of the lipids used). Sonication was performed using a MSE Mark II 150-watt ultrasonic disintegrator set at 10 μ peak to peak amplitude. After sonication no pH adjustment was needed. Phospholipid concentrations were determined by phosphate analysis according the method of Böttcher *et al.* (27). The vesicle preparations described above were used throughout our experiments and were chosen because they exhibit excellent clot-promoting activity (28).

Measurement of Rates of Thrombin Formation—Factor X_a either alone or in the presence of phospholipids, $CaCl_2$, and/or factor V_a was incubated for 3 to 5 min at 37°C in a buffer containing 20 mM Tris, 100 mM NaCl, and 0.5 mg/ml of ovalbumin at pH 7.5. Prothrombin was added and after different time intervals samples were taken and added to a cuvette (thermostated at 37°C), containing 30 μg of STI and 0.47 μmol of S 2238 in such an amount of the above buffer that the final volume became 2 ml. The amount of STI is sufficient to inhibit further conversion of prothrombin and to inhibit the low amidase activity of factor X_a with S 2238. It does, however, not affect the rate of conversion of S 2238 by thrombin. From the absorbance change at 405 nm, recorded on a Gilford spectrophotometer, the amount of thrombin is calculated from a calibration curve made with known amounts of thrombin. The calibration curve was determined under the assay conditions described above. The rate of thrombin formation in the incubation mixture is calculated from the amounts of thrombin present in the samples taken after different time intervals. Pure prethrombin 1 and prethrombin 2 have negligible amidase activity toward S 2238 and, therefore, do not contribute to the absorbance changes measured. Ovalbumin (0.5 mg/ml) was included in all protein solutions and incubations in order to prevent inactivation of the proteins.

For the construction of Lineweaver-Burk plots the rate of thrombin formation was averaged from three independent determinations. K_m, V_{max}, and the relevant standard errors were determined using the weighted and nonlinear regression method described by Wilkinson (29). Lines were drawn accordingly.

Gel Electrophoretic Analysis of Prothrombin Activation—Gel electrophoresis in the presence of sodium dodecyl sulfate was carried out as described by Laemmli (30) on gels containing 10% acrylamide, 0.27% *N,N'*-methylenebisacrylamide, and 0.1% sodium dodecyl sulfate. Aliquots (10 to 50 μl) of the incubation mixtures were added to 50 μl of 2% sodium dodecyl sulfate, 50 mM EDTA (pH 7.5) and kept for 4 min in a boiling water bath. Five per cent mercaptoethanol was present in disulfide-reduced gel samples.

RESULTS

Introductory Experiments to Determine the Conditions for the Measurements of the Kinetic Parameters—To allow a kinetic approach it is necessary to confirm that the rate of thrombin formation is constant in time and proportional to the amount of factor X_a present for all different compositions of the prothrombin-activating mixtures used. That this is the case is shown in Fig. 1 where are plotted the amounts of thrombin formed at different time intervals after starting the reaction with varying known amounts of factor X_a. Also when phospholipid or factor V_a, or both, are present the rate of thrombin formation is constant for a long period (measured up to 20 min) and is proportional to the amount of factor X_a over the entire range of factor X_a concentrations used in the further experiments (data not shown). The latter result indicates that either a constant fraction of factor X_a or all factor X_a added is bound to the phospholipid vesicles. Since the maximal rate of thrombin formation increases only 4-fold when the phospholipid concentration is increased from 2.6 μM to 240 μM (Fig. 11, to be discussed below) we conclude that at the low phospholipid concentrations 25% of the added

[2] The abbreviations used are: S 2238, H-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide dihydrochloride; S 2222, *N*-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-*p*-nitroanilide hydrochloride; *p*-NPGB, *p*-nitrophenyl-*p'*-guanidinobenzoate hydrochloride; RVV-X, purified factor X activator from Russell's viper venom; STI, soybean trypsin inhibitor.

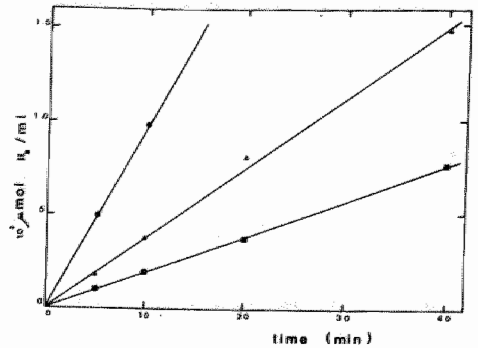


FIG. 1. **Time course of thrombin formation with different amounts of factor X_a.** Prothrombin (30 μM) was incubated with varying amounts of factor X_a in 1 ml of a buffer containing 20 mM Tris-HCl, 100 mM NaCl, 20 mM $CaCl_2$, and 0.5 mg/ml of ovalbumin (pH 7.5) at 37°C. After the time intervals indicated a sample was taken and assayed for the amount of thrombin as described under "Experimental Procedures." The amounts of factor X_a present were: ■——■, 9.1×10^{-5} μmol/ml; ▲——▲, 18.2×10^{-5} μmol/ml; and ●——●, 45.6×10^{-5} μmol/ml.

factor X_a is bound to the phospholipid vesicles, while at the higher phospholipid concentrations almost all factor X_a added is bound. The linearity of thrombin formation with time was preserved for all prothrombin concentrations used throughout.

It is well known that the rate of thrombin formation is influenced by the presence of factor V_a and Ca^{2+} (7, 13). In order to compare rates in the presence or absence of the accessory compounds it is necessary that the experiments be carried out under optimal conditions with respect to the amounts of these components. This was accomplished by measuring thrombin formation at different factor V_a and Ca^{2+} concentrations at constant amounts of factor X_a and prothrombin. Fig. 2 shows the Ca^{2+} titration of thrombin formation when factor X_a is converting prothrombin in solution in the presence and absence of factor V_a. The shapes of the titration curves and the optimal Ca^{2+} concentrations are independent of prothrombin, factor X_a, and factor V_a in the concentration range used in our further experiments (not shown). The significant inhibition at Ca^{2+} concentrations above 4 mM observed in the presence of factor V_a remains as yet unexplained. Similar Ca^{2+} titrations have been carried out for the prothrombinase complex consisting of phospholipids and factor X_a either in the absence or presence of factor V_a. Fig. 3 shows the Ca^{2+} dependence of the rate of thrombin formation at two different phospholipid concentrations (7.5 and 75 μM) in the absence of factor V_a. The same experiment carried out in the presence of factor V_a is shown in Fig. 4. It is interesting to note that in the presence of factor V_a and phospholipid the Ca^{2+} titration curves are sigmoidal.

The dependence of the thrombin formation rate on the amount of factor V_a present was measured at the optimal Ca^{2+} concentrations determined in the above mentioned experiments. When factor X_a converts prothrombin in solution in the presence of 3 mM $CaCl_2$, large amounts of factor V_a have to be added in order to obtain the optimal rate of thrombin formation (Fig. 5). The true maximal rate cannot be obtained experimentally, however, since thrombin formation is inhibited at high factor V_a concentrations. Much lower amounts of factor V_a are required to obtain an optimal rate of thrombin formation in the presence of phospholipids (Fig. 6). In this case no inhibitory effect of factor V_a is found, so the rates of thrombin formation in the presence of phospholipid and saturating amounts of factor V_a can be regarded as true optimal rates of prothrombin activation. In the presence of a 10-fold higher phospholipid concentration the amount of factor V_a needed to obtain half-saturation is increased about 5 times, and the optimal rate attained is about 15% lower, which is caused by a difference in the K_m for prothrombin at high and low phospholipid concentrations (see below).

Determination of the Kinetic Constants of Prothrombin Conversion with Different Prothrombin Activation Mixtures—Rates of thrombin formation were measured at varying prothrombin concentrations with different activation mixtures. When Ca^{2+} or factor V_a, or both, are present, the data

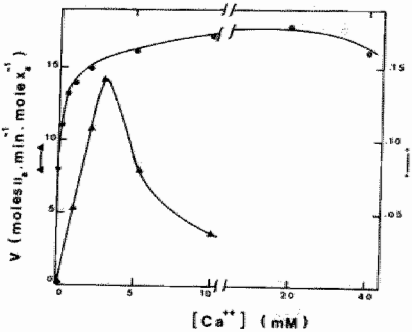


FIG. 2. **The Ca^{2+} dependence of thrombin formation in the absence and presence of factor V_a.** ●——●, prothrombin (30 μM) was incubated at 37°C with factor X_a (9.1×10^{-5} μmol/ml) in 1 ml of a buffer containing 20 mM Tris-HCl, 100 mM NaCl, 0.5 mg/ml of ovalbumin (pH 7.5), and varying amounts of $CaCl_2$. The rate of thrombin formation was calculated from the amounts of thrombin present after 5 and 10 min of incubation. ▲——▲, prothrombin (1.12 μM) was incubated with factor X_a (7.75×10^{-7} μmol/ml) and factor V_a (3.6 units/ml). Further experimental conditions as described above.

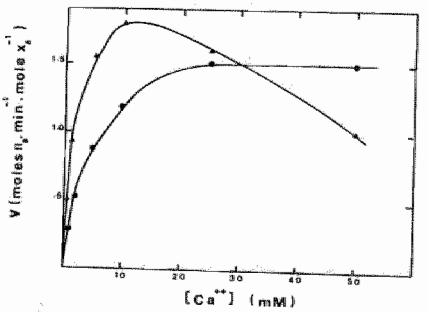


FIG. 3. **The Ca^{2+} dependence of the rate of thrombin formation in the presence of phospholipid.** Factor X_a (3.1×10^{-6} μmol/ml) was incubated at 37°C in 4 ml of a mixture containing 20 mM Tris-HCl, 100 mM NaCl, ovalbumin (0.5 mg/ml), 7.5 μM phospholipid (▲——▲) or 75 μM phospholipid (●——●), and varying amounts of $CaCl_2$ at pH 7.5. After 3 min the reaction was started by adding 40 μl of prothrombin, resulting in a final concentration of 0.25 μM. From the amounts of thrombin present after 5 and 10 min the rate of thrombin formation was calculated. The phospholipid vesicles were prepared as described under "Experimental Procedures."

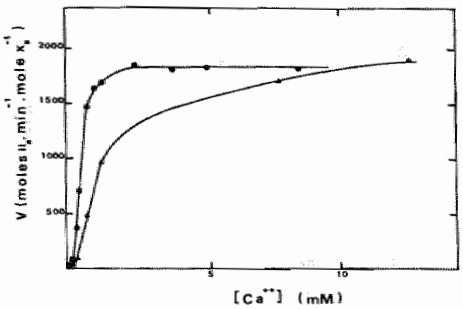


FIG. 4. **The Ca^{2+} dependence of the rate of thrombin formation in the presence of phospholipid and factor V_a.** Factor X_a (3.1×10^{-9} μmol/ml) was incubated at 37°C in 4 ml of a mixture containing 20 mM Tris-HCl, 100 mM NaCl, ovalbumin (0.5 mg/ml), 0.95 unit of factor V_a, 7.5 μM phospholipid (▲——▲) or 75 μM phospholipid (●——●), and varying amounts of $CaCl_2$ at pH 7.5. After 3 min the reaction was started by adding 40 μl of prothrombin giving a final concentration of 3 μM. For further experimental details see Fig. 3.

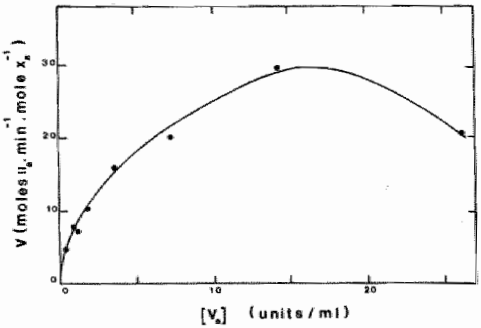


FIG. 5. **The factor V_a dependence of the rate of thrombin formation in solution.** Factor X_a (7.75×10^{-7} μmol/ml) was incubated at 37°C in 1 ml of a mixture containing 20 mM Tris-HCl, 100 mM NaCl, ovalbumin (0.5 mg/ml), 3 mM $CaCl_2$, and varying amounts of factor V_a at pH 7.5. After 5 min the reaction was started by addition of 10 μl of prothrombin to give a final concentration of 1.12 μM. From the amounts of thrombin present after 5 and 10 min of reaction time the rate of thrombin formation was calculated.

from Figs. 2 to 6 have been used to select concentrations that give an optimal rate of thrombin formation. In order to make a direct comparison of the different Lineweaver-Burk plots possible, rates of thrombin formation were expressed as moles of thrombin formed per min per mol of factor X_a present. From the kinetic data the K_m for prothrombin (in μM) and the V_{max} of thrombin formation (in moles of thrombin per min per mol of X_a) were determined. Fig. 7 shows the Lineweaver-Burk plot of factor X_a conversion of prothrombin into thrombin in the absence of accessory components. From this plot a K_m of 131 μM and a V_{max} of 0.61 mol min^{-1} mol X_a^{-1} can be calculated. When the same experiment is carried out in the presence of 20 mM Ca^{2+} there is a small decrease of the K_m while the V_{max} is not affected. With a prothrombin activation mixture consisting of factor X_a, Ca^{2+}, and factor V_a, a K_m of 34 μM is found (Fig. 8). In the presence of factor V_a the maximal rate of thrombin formation is increased about 600-fold to a value of 373 mol min^{-1} mol X_a^{-1}. Lineweaver-Burk plots of thrombin formation in the presence of phospholipid are shown in Fig. 9 and 10. With a prothrombin activation mixture consisting of factor X_a, phospholipid (7.5 μM), and Ca^{2+}, a V_{max} of 2.25 mol of thrombin min^{-1} mol X_a^{-1} and a K_m of 0.058 μM are found (Fig. 9). Increasing the phospholipid concentration slightly increases the V_{max}, and a much higher K_m value is measured. Compared with the kinetic parameters measured under the same conditions, but in the absence of phospholipids, the considerable decrease of K_m values is most striking. Fig. 10 shows the Lineweaver-Burk plot of the complete prothrombinase complex (factor X_a, phospholipid, Ca^{2+}, and factor V_a). As expected this prothrombin activation mixture yields kinetic constants which greatly favor thrombin formation. A low K_m value for prothrombin, which is again dependent on the phospholipid concentration, and a high V_{max} of

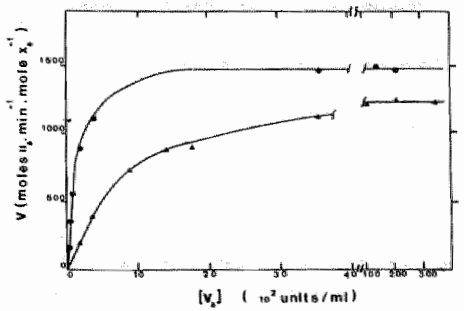


FIG. 6. **The effect of factor V_a on the rate of thrombin formation in the presence of phospholipid.** Factor X_a (2.3×10^{-9} μmol/ml) was incubated at 37°C in 4 ml of a mixture containing 20 mM Tris-HCl, 100 mM NaCl, ovalbumin (0.5 mg/ml), 7.5 μM phospholipid (●——●) or 75 μM phospholipid (▲——▲), 5 mM $CaCl_2$, and varying amounts of factor V_a at pH 7.5. After 3 min the reaction was started by adding 60 μl of prothrombin resulting in a final concentration of 0.95 μM. From the amounts of thrombin present after 4 and 8 min of reaction time, the rate of thrombin formation was calculated.

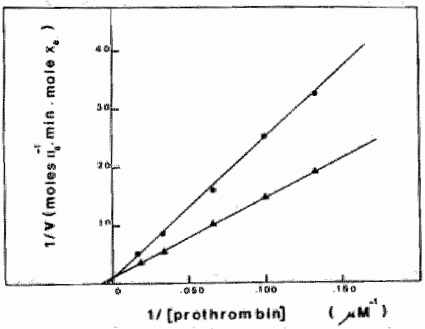


FIG. 7. **Lineweaver-Burk plot of factor X_a converting prothrombin into thrombin in the absence and presence of $CaCl_2$.** Thrombin formation at varying concentrations of prothrombin was measured at 37°C in 1 ml of a reaction mixture containing 20 mM Tris-HCl, 100 mM NaCl, ovalbumin (0.5 mg/ml), factor X_a (9.1×10^{-5} μmol/ml) at pH 7.5 without $CaCl_2$ (●——●) and in the presence of 20 mM $CaCl_2$ (▲——▲). The reaction was started by addition of factor X_a. After 7½ and 15 min samples were taken and assayed for thrombin as described under "Experimental Procedures." From the amounts of thrombin found the rate of thrombin formation was calculated. The kinetic constants calculated are summarized in Table I.

1919 mol of thrombin min^{-1} mol X_a^{-1} at 7.5 μM phospholipid can be calculated from the experimental data. Table I summarizes the kinetic constants for thrombin formation with different activation mixtures calculated from the data presented in Figs. 7 to 10.

The dependence of the kinetic parameters on the amount of phospholipid present is studied in more detail for the prothrombin-activating complex consisting of factor X_a, phospholipid, and Ca^{2+} (Table II). The gradual increase of the K_m

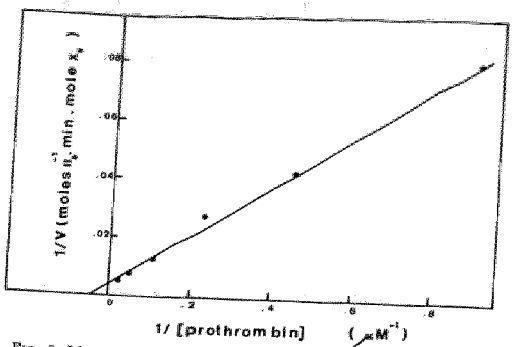


FIG. 8. **Lineweaver-Burk plot of factor X_a converting prothrombin into thrombin in the presence of factor V_a and $CaCl_2$.** Thrombin formation at varying prothrombin concentrations was measured at 37°C in 1 ml of a reaction mixture containing 20 mM Tris-HCl, 100 mM NaCl, ovalbumin (0.5 mg/ml), 3 mM $CaCl_2$, factor X_a (7.75×10^{-7} μmol/ml), and factor V_a (3.5 U/ml) at pH 7.5. The reaction was started by addition of prothrombin after 5 min of preincubation. Further experimental details are as in Fig. 7. The kinetic constants found are summarized in Table I.

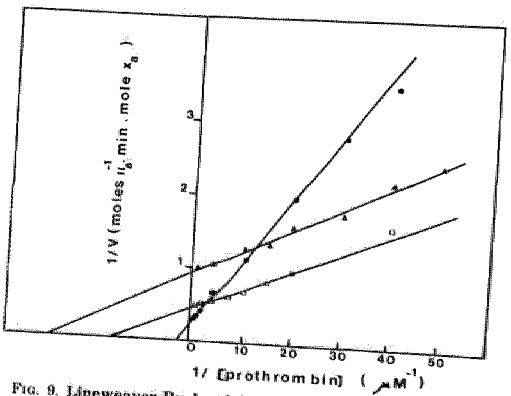


FIG. 9. **Lineweaver-Burk plot of factor X_a converting prothrombin into thrombin in the presence of phospholipid.** Thrombin formation at varying prothrombin concentrations was measured at 37°C in 4 ml of a reaction volume containing 20 mM Tris-HCl, 100 mM NaCl, ovalbumin (0.5 mg/ml), factor X_a (3.87×10^{-7} μmol/ml), 2.6 μM phospholipid and 8 mM $CaCl_2$ (▲——▲), 7.5 μM phospholipid and 15 mM $CaCl_2$ (□——□) or 75 μM phospholipid and 25 mM $CaCl_2$ (●——●) at pH 7.5. The reaction was started after 3-min preincubation by the addition of prothrombin. At 4 and 8 min after prothrombin addition samples were taken from the reaction mixture and assayed for thrombin in order to calculate the rate of thrombin formation. The kinetic constants calculated are summarized in Table I.

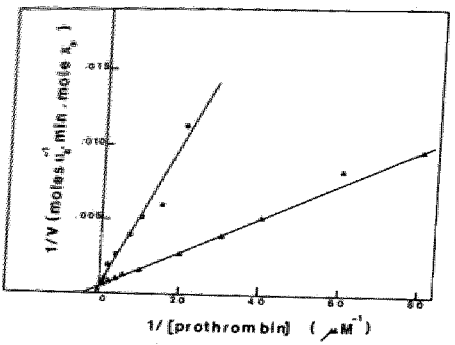


FIG. 10. **Lineweaver-Burk plot of factor X_a converting prothrombin into thrombin in the presence of phospholipid and factor V_a.** The experimental details are described in the legend of Fig. 9 except the amount of factor X_a was 7.75×10^{-10} μmol/ml, and 0.9 U/ml of factor V_a was present. The $CaCl_2$ concentrations were 7.5 mM and 5.0 mM at, respectively, 7.5 μM phospholipid (▲——▲) and 75 μM phospholipid (●——●).

TABLE I

Kinetic constants of thrombin formation with various prothrombin-activating mixtures

Prothrombin-activating mixture	K_m for prothrombin	V_{max}
	μM ± S.E.	*mol II_a min^{-1} mol X_a^{-1}* ± S.E.
Factor X_a	131 ± 24	0.61 ± 0.08
Factor X_a, $CaCl_2$	84 ± 11	0.68 ± 0.06
Factor X_a, $CaCl_2$, factor V_a	34 ± 5	373 ± 30
Factor X_a, $CaCl_2$, phospholipid (2.6 μM)	0.032 ± 0.003	1.06 ± 0.05
Factor X_a, $CaCl_2$, phospholipid (7.5 μM)	0.058 ± 0.005	2.25 ± 0.05
Factor X_a, $CaCl_2$, phospholipid (75 μM)	0.35 ± 0.03	3.9 ± 0.1
Factor X_a, $CaCl_2$, phospholipid (7.5 μM), factor V_a	0.21 ± 0.02	1919 ± 63
Factor X_a, $CaCl_2$, phospholipid (75 μM), factor V_a	1.7 ± 0.6	2748 ± 580

for prothrombin with increasing phospholipid concentrations is obvious. The increase of the V_{max} of the thrombin formation at higher amounts of phospholipid is much less pronounced. This is likely due to the fact that the V_{max} is determined by the amount of factor X_a actually bound to the phospholipid at the different phospholipid concentrations. The kinetic parameters of free factor X_a (Table I) are such that any nonbound factor X_a has no detectable contribution to thrombin formation in the presence of phospholipid. Thus variation of V_{max} with the phospholipid concentration is determined by the binding isotherm of factor X_a to varying amounts of phospholipid. Extrapolation of a double reciprocal plot ($1/V_{max}$ *versus* 1/[phospholipid]) at constant factor X_a to infinite phospholipid concentration yields the V_{max} for the case that all factor X_a is bound (Fig. 11). The V_{max} calculated from this plot is 4 mol min^{-1} mol X_a^{-1}, which shows that even at the lowest phospholipid concentration used in our experiments about 25% of the added factor X_a is bound to the phospholipid vesicles.

Time Course of Prothrombin Activation by Different Prothrombin Activation Mixtures Visualized by Sodium Dodecyl Sulfate Gel Electrophoresis—The experiments of the group of Jackson (2–7) have shown that different partial prothrombin activation products accumulate during activation of prothrombin with different activation mixtures. It can be argued

TABLE II

Dependence of K_m and V_{max} on the phospholipid concentration

Prothrombin was activated by factor X_a in the presence of Ca^{2+} and phospholipid. Reactions were carried out in a mixture containing 4.6×10^{-7} μmol of X_a/ml, 100 mM NaCl, 25 mM Tris, 0.5 mg of ovalbumin/ml at 37°C and pH 7.5. Amounts of phospholipid and $CaCl_2$ present are indicated in the table. Further experimental details are described in the legend to Fig. 9 and under "Experimental Procedures."

Phospholipid	$CaCl_2$[a]	K_m	V_{max}
μM	mM	μM ± S.E.	mol II_a min^{-1} mol X_a^{-1} ± S.E.
2.6	8	0.032 ± 0.003	1.06 ± 0.05
4.0	8	0.062 ± 0.005	1.27 ± 0.04
5.3	10	0.054 ± 0.003	1.54 ± 0.03
7.5	10	0.058 ± 0.005	2.25 ± 0.05
8.0	10	0.09 ± 0.02	2.04 ± 0.20
10.5	10	0.068 ± 0.008	1.75 ± 0.09
16.0	10	0.14 ± 0.01	2.35 ± 0.08
26.3	15	0.164 ± 0.006	2.71 ± 0.04
40.0	15	0.23 ± 0.02	3.12 ± 0.10
52.6	25	0.25 ± 0.01	2.90 ± 0.07
75	25	0.35 ± 0.03	3.90 ± 0.10
80	25	0.46 ± 0.01	3.52 ± 0.03
105	32	0.48 ± 0.05	3.31 ± 0.20
240	40	1.08 ± 0.07	4.10 ± 0.10

[a] For each phospholipid concentration a Ca^{2+} titration was performed to obtain the optimal Ca^{2+} concentration.

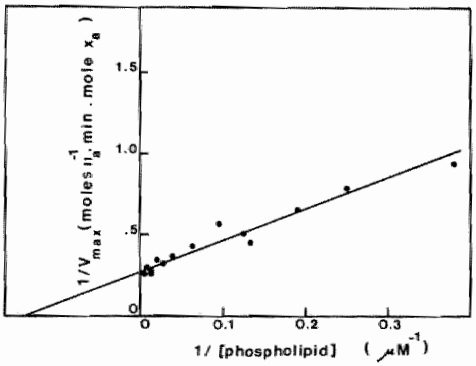


FIG. 11. **Double reciprocal plot of V_{max} as a function of the phospholipid concentration.** The prothrombin activating mixture consisted of factor X_a, $CaCl_2$, and phospholipid. This plot contains data summarized in Table II.

that the kinetic parameters measured in the experiments described above are in fact those for a mixture of prothrombin and its partial activation products liberated during the time course of the reaction. However, gel electrophoretic analysis of the products formed during prothrombin activation under the conditions of our experiments shows that only a negligible amount of prothrombin is converted into thrombin and other partial activation products. Preliminary experiments[3] on the kinetics of prethrombin 1 and prethrombin 2 point out that a possible conversion of liberated activation products cannot contribute to thrombin formation in our experiments. To investigate whether the changes of kinetic parameters are accompanied or caused by changes in the reaction pathway of prothrombin activation it is necessary to devise experiments in which sufficient activation products are formed. By increasing the amount of enzyme (factor X_a and factor V_a, if present) it is possible to follow prothrombin activation by different prothrombin-activating mixtures using sodium dodecyl sulfate gel electrophoresis (Fig. 12).

Prothrombin and its activation products are identified in the figures. The relative migration distances and the sequence of prothrombin and activation products on the gel are consistent with earlier published gel data (2–6). In the absence of factor V_a (with or without phospholipid) prethrombin 2 is the main end product during the initial phase of prothrombin activation (Fig. 12, *A* and *C*). At a later stage of the activation process sufficient thrombin is formed to be detectable on the gels. With factor V_a in the absence of phospholipid, thrombin is generated in excess of prethrombin 2 (Fig. 12*B*), whereas in the presence of phospholipid and factor V_a formation of prethrombin 2 cannot be detected (Fig. 12*D*). These data point out that in the presence of factor V_a a shift in the pathway of prothrombin activation occurs (see under "Discussion"). Formation of prethrombin 1 during prothrombin activation will take place as a result of thrombin-catalyzed proteolysis of prothrombin. Indeed prethrombin 1 is formed during the time course of prothrombin activation. In particular, it is found in those experiments where considerable amounts of thrombin are formed.

DISCUSSION

The studies of Esmon *et al.* (7) and Jobin and Esnouf (13) have shown that both phospholipid and factor V_a increase the rate of conversion of prothrombin to thrombin by factor X_a. Since their experiments were carried out at a single concentration of prothrombin, factor X_a, factor V_a, and phospholipid, no insight in the mechanistic function of factor V_a and phospholipid in the prothrombinase complex could be obtained. Several explanations can be proposed for the rate enhancements observed in the presence of the accessory components of the prothrombinase complex (*cf.* Ref. 1) *e.g.*: 1. a change in the reaction pathway of prothrombin activation; 2. an increase of the proteolytic capacity of factor X_a upon interaction with phospholipid, factor V_a, and/or Ca^{2+}; 3. a conformational change of prothrombin upon binding to phospholipid, factor V_a, and/or Ca^{2+}, making it a more readily cleavable substrate; 4. an increase of local prothrombin and factor X_a concentration after binding to phospholipid and/or factor V_a promoting the formation of the prothrombin-prothrombinase complex; 5. binding of prothrombin, factor V_a, and factor X_a to phospholipid in a favorable steric position. It is obvious that these different mechanisms may act simultaneously. None of these possibilities can be excluded on the basis of the properties of the prothrombinase complex reported in the literature.

Knowledge of the effects of phospholipid and factor V_a on the kinetic parameters of prothrombin activation will be a prerequisite in order to elucidate their role in the mechanism of the prothrombinase complex.

The kinetic parameters of different prothrombin-activating mixtures are summarized in Table I. In the absence of accessory components prothrombin is a very poor substrate for factor X_a. The high K_m value (131 μM) indicates that prothrombin has a low affinity for factor X_a under these conditions. Taking into account a plasma prothrombin concentration of about 1.5 μM, which is far below the K_m, and the low V_{max} of thrombin formation measured under these conditions, it is unlikely that physiologically significant thrombin formation can take place without involvement of phospholipid and factor V_a.

The small decrease of the K_m for prothrombin occurring when the same experiment is carried out in the presence of Ca^{2+} may be the result of Ca^{2+} binding to γ-carboxyglutamic acid residues present in factor X_a and prothrombin. Since

[3] J. W. P. Govers-Riemslag and J. Rosing, unpublished observations.

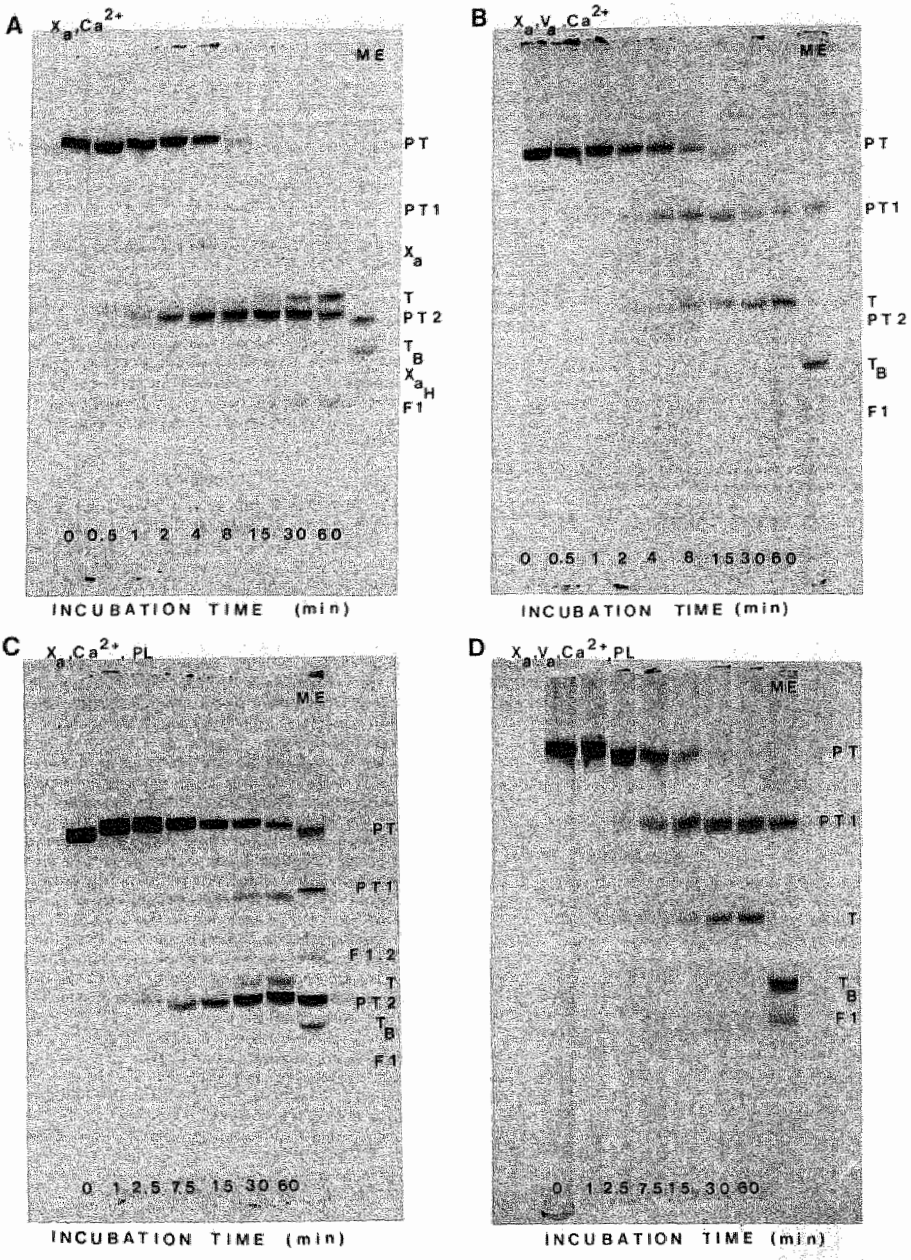


FIG. 12. **Time course of activation of prothrombin with different activating mixtures.** *A*, prothrombin (13.4 μM) was activated with factor X_a (1.82 μM) in a buffer containing 100 mM NaCl, 25 mM Tris, 2 mM diisopropylphosphorofluoridate and 10 mM $CaCl_2$ at 37°C and pH 7.5. *B*, prothrombin (13.4 μM) was activated with factor X_a (6.2×10^{-3} μM) and factor V_a (19 units/ml) in a buffer containing 100 mM NaCl, 25 mM Tris, 2 mM diisopropylphosphorofluoridate, and 3 mM $CaCl_2$ at 37°C and pH 7.5. *C*, prothrombin (2.68 μM) was activated

Ca^{2+} has no effect on the V_{max} of thrombin formation we conclude that the catalytic efficiency (k_{cat}/K_m) of factor X_a is virtually not changed upon Ca^{2+} binding.

Important changes of kinetic parameters take place when phospholipid or factor V_a, or both, form part of the prothrombinase complex. Phospholipid causes a profound decrease of the K_m for prothrombin. In the presence of phospholipid the K_m decreases to values below 1 μM, which is lower than the plasma prothrombin concentration.

The K_m for prothrombin is, however, dependent on the amount of phospholipid present (Table II). Higher K_m values are measured at increasing phospholipid concentrations. The K_m increases from 0.032 μM at 2.6 μM phospholipid to 1.08 μM at a phospholipid concentration of 240 μM. Therefore, a K_m determined in the presence of phospholipid has to be regarded as an apparent K_m. This is not surprising since the K_m value is calculated using the concentrations of total added prothrombin. However, the thrombin formed at the phospholipid surface is in fact generated from bound prothrombin. The prothrombin concentration at the K_m should, therefore, be expressed in terms of surface concentration in those experiments where K_m values at different phospholipid concentrations are compared. To calculate the amount of bound prothrombin, binding parameters (*e.g.* available binding sites and dissociation constants) have to be known for prothrombin binding to the phospholipid vesicles under our experimental conditions (temperature, pH, ionic strength, and $[Ca^{2+}]$). Although the appropriate binding data are not available, we used as a first approximation prothrombin binding parameters of Nelsestuen and Broderius (31). For conditions approaching those of our experiments as close as possible (0.05 M Tris, 0.1 M NaCl, 5 mM $CaCl_2$ at pH 7.5 and 25°C) they reported a dissociation constant of 10^{-7} M for the prothrombin·phospholipid complex and 17 μmol of prothrombin binding sites per g of phospholipid for vesicles prepared from a 1/1 (mol/mol) mixture of bovine brain phosphatidylserine and egg yolk phosphatidylcholine. Using these binding data we calculated the prothrombin density at the phospholipid surface, expressed as micromoles of prothrombin bound per g of phospholipid, at the K_m measured at different phospholipid concentrations (Table III). Although the apparent K_m increases when increasing amounts of phospholipid are present, the prothrombin density at the phospholipid surface at the K_m is independent of the phospholipid concentration. This means that it is the local prothrombin concentration at the phospholipid surface which determines the kinetics in this case. Our experiments allow no conclusion as to what extent an increased local prothrombin concentration explains the large decrease of the K_m observed in the presence of phospholipid. This implies also that the possibility that phospholipid brings prothrombin and factor X_a together in a more favorable orientation, the so-called juxtaposing effect, cannot be evaluated. Legitimate conclusions in this connection have to be based on a theoretical treatment of enzyme kinetics in solution and heterogeneous enzyme catalysis taking place at the phospholipid surface. This requires, for instance, knowledge of diffusion constants in solution and at the phospholipid surface, appropriate binding data, and orientation at the phospholipid surface of the proteins involved.

TABLE III

K_m for prothrombin and corresponding prothrombin density at the phospholipid surface at varying phospholipid concentrations

Phospholipid	K_m	Prothrombin density at phospholipid surface
μM	μM	μmol/g ± S.E.
2.6	0.032	3.4 ± 0.3
4.0	0.062	5.2 ± 0.5
5.3	0.054	4.4 ± 0.3
7.5	0.058	4.2 ± 0.4
8.0	0.090	5.7 ± 1.0
10.5	0.068	4.2 ± 0.5
16.0	0.14	6.2 ± 0.6
26.3	0.164	5.5 ± 0.2
40.0	0.23	5.7 ± 0.6
52.6	0.25	5.4 ± 0.3
75	0.35	5.1 ± 0.4
80	0.46	6.2 ± 0.1
105	0.48	5.2 ± 0.5
240	1.08	5.4 ± 0.4

The role of factor V_a in the complete prothrombinase complex (factor X_a, factor V_a, Ca^{2+}, and phospholipid) is mainly restricted to an effect on the V_{max} of thrombin formation. A 700-fold increase of V_{max} is observed in the presence of factor V_a.

With respect to the mode of action of factor V_a in the prothrombinase complex one has to consider whether factor V_a interacts with prothrombin, changing its properties as a substrate or whether it forms a complex with factor X_a with a catalytic capacity different from free factor X_a. We exclude the possibility that factor V_a exerts its stimulatory action, independent of factor X_a or prothrombin, by trapping potential inhibitory activation peptides released during prothrombin activation. Neither fragment 1, fragment 2, nor fragment 1.2 inhibit thrombin formation at concentrations generated during prothrombin activation in the time course of our experiments. Since the factor V_a concentration in our experiments is always much lower than the prothrombin concentration a prothrombin·factor V_a complex, if present, will be a small fraction of the total amount of prothrombin added. This combined with the fact that a further increase of the amount of factor V_a does not affect the kinetics of the reaction makes it very unlikely that a prothrombin·factor V_a complex acts as a substrate for factor X_a. Therefore, it seems plausible to assume that a factor X_a·factor V_a complex is the catalytic unit in the prothrombinase complex.

In solution factor V_a also exhibits a stimulating effect on thrombin formation. However, from the experiment shown in Fig. 5 it is clear that it is impossible to achieve complete saturation of factor X_a with factor V_a. At high concentrations factor V_a even inhibits thrombin formation. This phenomenon, for which we have no explanation yet, prevents adding saturating amounts of factor V_a. This leaves open the question whether phospholipid actually has an additional effect on the rate enhancement of thrombin formation brought about by factor V_a.

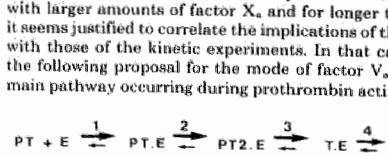
with factor X_a (5.5×10^{-3} μM) in a buffer containing 100 mM NaCl, 25 mM Tris, 2 mM diisopropylphosphorofluoridate, 75 μM phospholipid, and 25 mM $CaCl_2$ at 37°C and pH 7.5. *D*, prothrombin (2.68 μM) was activated with factor X_a (6.2×10^{-5} μM) and factor V_a (10 units/ml) in a buffer containing 100 mM NaCl, 25 mM Tris, 2 mM diisopropylphosphorofluoridate, 75 μM phospholipid, and 5 mM $CaCl_2$ at 37°C and pH 7.5. In order to slow down prothrombin conversion by thrombin formed 2 mM diisopropylphosphorofluoridate was included in the activation mixture. Time points at which samples were taken and the identification of the band pattern are indicated in the figure. Abbreviations used are: *PL*, phospholipid; *PT*, prothrombin; *PT1*, prethrombin 1; X_a, factor X_a; *F1.2*, fragment 1.2; *T*, thrombin; *PT2*, prethrombin 2; T_B, B chain of thrombin; X_aH, heavy chain of factor X_a; and *F1*, fragment 1. For further experimental details see under "Experimental Procedures." The last gel of each series (indicated by *ME*) is a disulfide-reduced sample of the reaction mixture taken after 60 min of incubation. For this gel β-mercaptoethanol is used as reducing agent.

Comparison of the factor V_a dependence of thrombin formation in the absence and presence of phospholipid (Figs. 5 and 6) draws attention to another function of phospholipids in the prothrombinase complex. Since much lower amounts of factor V_a are required in the presence of phospholipid to maximize the rate of thrombin formation, we conclude that phospholipids promote the formation of the factor V_a-X_a complex. This may be caused by increased local factor V_a and factor X_a concentrations at the phospholipid surface after binding of both proteins to the vesicles. This will shift the equilibrium of formation of the factor V_a-X_a complex in the direction of association. For a discussion of the actual mechanism that causes the increased V_{max} of thrombin formation in the presence of factor V_a it is helpful to consider Scheme 1, depicting a minimal mechanism for the conversion of prothrombin and other activation products.

The different proteins and complexes represented in this scheme are: *E*, prothrombin activating complex; *PT*, prothrombin; *PT1*, prethrombin 1; *PT2*, prethrombin 2; *T*, thrombin; *F1*, prothrombin fragment 1; *F2*, prothrombin fragment 2; *F1.2*, prothrombin fragment 1.2.

The reactions represented in this scheme are based on results reported in the literature (2–7, 32). Although thrombin formation can take place with free prethrombin 1 or prethrombin 2 as substrate (5), electrophoretic analysis shows that in the course of our kinetic experiments such small amounts of partial activation products accumulate that conversion of intermediates released from the prothrombinase complex or formed by the action of thrombin cannot contribute to the amount of thrombin formed. Therefore, thrombin is generated in our kinetic experiments via the pathway indicated by the *solid arrows* (Steps 1, 2, 3, 4).

An intriguing explanation for the mechanism of rate enhancement by factor V_a can be put forward on the basis of the experiments (Fig. 12) in which the activation of prothrombin with different prothrombin-activating mixtures is followed by sodium dodecyl sulfate gel electrophoresis. The experimental setup is a refinement of similar experiments carried out by the group of Jackson (2, 4, 5, 6). Our reaction conditions, prothrombin concentration, and composition of activating mixtures are chosen on the basis of knowledge obtained from our kinetic experiments. When factor X_a converts prothrombin in the absence and presence of phospholipid, but in the absence of factor V_a, mainly prethrombin 2 is formed, while with the complete prothrombinase complex (factor X_a, factor V_a, phospholipid, and Ca^{2+}) thrombin is the main end product, and no prethrombin 2 is detectable. Trace amounts of prethrombin 2 are formed when factor X_a activates prothrombin in the presence of factor V_a and Ca^{2+}. Since it is not possible to saturate factor X_a with factor V_a under these conditions (Fig. 5) the small amounts of prethrombin 2 are likely formed by free factor X_a. Although such experiments are carried out with larger amounts of factor X_a and for longer time periods, it seems justified to correlate the implications of these findings with those of the kinetic experiments. In that case we make the following proposal for the mode of factor V_a action. The main pathway occurring during prothrombin activation in the absence of factor V_a, either in the absence or presence of phospholipid, is that giving rise to prethrombin 2 formation (steps 1, 2, 5). Only a small fraction of prothrombin is converted into thrombin (Steps 1, 2, 3, 4) and is measured in the kinetic experiments. Prethrombin 2 is the main end product since it easily dissociates from factor X_a (step 5). Reassociation with factor X_a, which offers a second chance to be converted to thrombin, is inhibited by the large excess of prothrombin present. Factor V_a changes the pathway of prothrombin activation from one resulting in prethrombin 2 (steps 1, 2, 5) into one giving rise to thrombin (steps 1, 2, 3, 4). Apparently the presence of factor V_a prevents the dissociation of prethrombin 2 from the prothrombinase complex. The tight association between fragment 1.2 and both prethrombin 2 and factor V_a (5) can explain why prethrombin 2 does not dissociate from the prothrombinase complex. Dissociation of prethrombin 2 can, however, also be prevented when factor V_a increases a limiting rate constant occurring in the reaction scheme after formation of the PT2.E complex. This will decrease the steady state concentration of the latter intermediate, with a consequent drop of the rate of dissociation of prethrombin 2. It must be emphasized that more complex mechanisms may be devised to accommodate our observations. However, the proposed explanation for the observed shift in the pathway brought about by factor V_a is consistent with our results and data available in the literature. A study on the kinetics of activation of partial prothrombin activation products with various activating mixtures and the effects of prothrombin fragments thereon, in progress in our laboratory, will lead to a more detailed description of the mechanism of prothrombin activation.



SCHEME 1

Our findings may have important implications for the mechanism of other phospholipid-protein complexes that participate in blood coagulation. The role of factor VIII and phospholipid in the factor X-activating complex consisting of factor IX_a, factor $VIII_a$, phospholipid, and Ca^{2+} and by extension, the role of the protein and phospholipid component of tissue thromboplastin in the extrinsic factor X activator (factor VII_a, tissue thromboplastin, and Ca^{2+}) may be identical with those of phospholipid and factor V_a in the prothrombinase complex. Indeed Silverberg *et al.* (14) reported that tissue factor increases the k_{cat} of factor X activation by factor VII_a about 2900-fold and decreased the K_m for factor X 10-fold. Since tissue factor contains both the phospholipid and protein accessory component it is not possible to separate their effect on the kinetic parameters. A kinetic study of the activation of factor X by factor IX_a in the absence and presence of factor $VIII_a$, phospholipid, and Ca^{2+} is in progress in our laboratory.

Acknowledgments—We would like to thank Dr. H. v. Zutphen for synthesis of the phospholipids, Dr. G. v. Dieyen for preparation of benzamidine-Sepharose and Mr. H. Bruls for technical assistance.

REFERENCES

1. Jackson, C. M., and Suttie, J. W. (1977) *Physiol. Rev.* **57**, 1–70
2. Owen, W. G., Esmon, C. T., and Jackson, C. M. (1974) *J. Biol. Chem.* **249**, 594–605
3. Esmon, C. T., Owen, W. G., and Jackson, C. M. (1974) *J. Biol. Chem.* **249**, 606–611
4. Esmon, C. T., and Jackson, C. M. (1974) *J. Biol. Chem.* **249**, 7782–7790
5. Esmon, C. T., and Jackson, C. M. (1974) *J. Biol. Chem.* **249**, 7791–7797
6. Esmon, C. T., Owen, W. G., and Jackson, C. M. (1974) *J. Biol. Chem.* **249**, 7798–7807
7. Esmon, C. T., Owen, W. G., and Jackson, C. M. (1974) *J. Biol. Chem.* **249**, 8045–8047
8. Papahadjopoulos, D. P., and Hanahan, D. J. (1964) *Biochim. Biophys. Acta* **90**, 436–439

9. Papahadjopoulos, D. P., Hougie, C., and Hanahan, D. J. (1962) *Proc. Soc. Exp. Biol. Med.* **111**, 412–416
10. Barton, P. G., and Hanahan, D. J. (1969) *Biochim. Biophys. Acta* **187**, 319–327
11. Kandall, C. L., Shohet, S. B., Akinbarni, T. K., and Colman, R. W. (1975) *Thromb. Diath. Haemorrh.* **34**, 271–284
12. Zwaal, R. F. A. (1978) *Biochim. Biophys. Acta* **515**, 163–205
13. Jobin, F., and Esnouf, M. P. (1967) *Biochem. J.* **102**, 666–674
14. Silverberg, S. A., Nemerson, Y., and Zur, M. (1977) *J. Biol. Chem.* **252**, 8481–8488
15. Di Scipio, R. G., Hermodson, M. A., Yates, S. G., and Davie, E. W. (1977) *Biochemistry* **16**, 698–706
16. Cuatrecasas, P. (1970) *J. Biol. Chem.* **245**, 3059–3065
17. Fujikawa, K., Legaz, M. E., and Davie, E. W. (1972) *Biochemistry* **11**, 4882–4891
18. Fujikawa, K., Legaz, M. E., and Davie, E. W. (1972) *Biochemistry* **11**, 4892–4899
19. Smith, R. L. (1973) *J. Biol. Chem.* **248**, 2418–2423
20. Schiffman, S., Theodor, J., and Rapaport, S. J. (1969) *Biochemistry* **8**, 1397–1405
21. Smith, C. M., and Hanahan, D. J. (1976) *Biochemistry* **15**, 1830–1838
22. Schmer, G. (1972) *Hoppe-Seyler's Z. Physiol. Chem.* **353**, 810–814
23. Chase, T., Jr., and Shaw, E. (1969) *Biochemistry* **8**, 2212–2224
24. Bear, E., and Buchnea, D. (1959) *Can. J. Biochem. Physiol.* **37**, 953–959
25. Comfurius, P., and Zwaal, R. F. A. (1977) *Biochim. Biophys. Acta* **488**, 36–42
26. De Kruijff, B., Cullis, P. R., and Radda, G. K. (1975) *Biochim. Biophys. Acta* **406**, 6–20
27. Böttcher, C. J. F., van Gent, C. M., and Pries, C. (1961) *Anal. Chim. Acta* **24**, 203–207
28. Tans, G., van Zutphen, H., Comfurius, P., Hemker, H. C., and Zwaal, R. F. A. (1979) *Eur. J. Biochem.* **95**, 449–457
29. Wilkinson, G. N. (1961) *Biochem. J.* **80**, 324–332
30. Laemmli, U. K. (1970) *Nature* **227**, 680–685
31. Nelsestuen, G. L., and Broderius, M. (1977) *Biochemistry* **16**, 4172–4177
32. Mann, K. G., Heldenbrand, C. M., Fass, D. N., Bajaj, S. P., and Butkowski, R. J. (1974) *Thromb. Diath. Haemorrh. Suppl.* **57**, 179–196

CHAPTER 5

THE ROLE OF PHOSPHOLIPID AND FACTOR VIII$_a$ IN THE ACTIVATION OF BOVINE FACTOR X

Gerbrand van Dieijen, Guido Tans, Jan Rosing and H.Coenraad Hemker

SUMMARY

The kinetic parameters of bovine factor X activation by bovine factor IX$_a$ have been determined in the absence and presence of Ca^{2+}, thrombin activated bovine factor VIII (VIII$_a$) and phospholipid (dioleoylphosphatidylcholine/dioleoylphosphatidylserine, 75/25; mole/mole). Factor IX$_a$ in the absence of Ca^{2+}, factor VIII$_a$ and phospholipid is able to catalyse factor X activation. The K_m for factor X is 299 μM which is well above its concentration in bovine plasma of about 0.2 μM. The V_{max} of factor X$_a$ formation is 0.0022 mole X$_a$.min^{-1}.mole IX$_a^{-1}$ under these conditions. Addition of Ca^{2+} has little effect on the kinetic constants of factor X activation by factor IX$_a$. In the presence of 10 mM $CaCl_2$ the K_m for factor X is 181 μM and the V_{max} is 0.0105 mole X$_a$.min^{-1}.mole IX$_a^{-1}$. The presence of 10 μM phospholipid dramatically decreases the K_m for factor X to 0.058 μM and the V_{max} becomes 0.0025 mole X$_a$.min^{-1}.mole IX$_a^{-1}$. The V_{max} of factor X$_a$ formation slightly increases when more phospholipid is present in our experiments and there is a considerable increase of the K_m for factor X at higher phospholipid concentrations. Therefore, the K_m measured in the presence of phospholipid has to be regarded as an apparent K_m. The possible explanations for this phenomenon are discussed.

For the complete factor X activating complex (i.e. factor IX$_a$, factor VIII$_a$, Ca^{2+} and 10 μM phospholipid), the K_m for factor X is 0.0063 μM and the V_{max} is raised 200,000 fold to 500 mole X$_a$.min^{-1}.mole IX$_a^{-1}$. In order to exert its stimulating effect on factor X activation, factor VIII has to be activated with thrombin. Our results show that factor IX$_a$ is an enzyme which can activate factor X at a very low rate. The stimulating effect of phospholipid in factor X activation is mainly due to an effect on the K_m for factor X, bringing it within the range of the plasma concentration. The stimulatory effect of factor VIII$_a$ is explained by its 50,000 fold increase of the V_{max} of factor X$_a$ formation.

INTRODUCTION

Blood coagulation factor X *) is a plasma glycoprotein that, during the clotting process, is converted into the serine protease factor X_a through proteolytic cleavage of a single peptide bond (1,2). Factor X_a is the enzyme that, during the coagulation process, activates prothrombin to thrombin in a reaction accelerated by factor V_a, calcium ions and phospholipid.

Under physiological conditions, the activation of factor X can be accomplished in both the extrinsic and intrinsic pathways of blood coagulation. In the extrinsic pathway, factor X is activated by a complex of factor VII, tissue factor and calcium ions (for a review, see ref. 3), whereas the activation of factor X via the intrinsic pathway involves the interaction of factor IX_a, factor VIII, calcium ions and phospholipid.

It is now generally accepted that factor IX_a, factor VIII, phospholipid and calcium ions form a complex (1, 8-11) in which factor IX_a likely is the enzyme responsible for factor X activation, since it is highly homologous to other serine proteases involved in blood coagulation (12,13), and factor VIII, phospholipid and calcium ions function as cofactors (14). Thus it is tempting to speculate that the roles of the components of the intrinsic factor X activator are analogous to those of factors X_a, V_a, phospholipid and calcium ions in the prothrombin activating complex (7,8,9,11,14).

Since the activity of factor VIII is considerably enhanced by preincubation with small amounts of thrombin, it is likely that factor VIII participates in the factor X activating complex in an activated form (15). Davie et al. (16,18) reported that factor VIII can also be activated with factor X_a. Of interest is the finding of Vehar and Davie (17,18) that thrombin activated factor VIII is inhibited by diisopropylphosphorofluoridate and antithrombin III which may have consequences for the way in which factor VIII functions in factor X activation.

In addition to the proteins factor IX_a and factor VIII, the factor X activating complex requires negatively charged phospholipid and calcium ions. It has been shown that the proteins have to be adsorbed to the phospholipid bilayer surface during the activation reaction (6-11, 14). Both vitamin K dependent clotting factors IX_a and X bind to the phospholipid surface via calcium bridges between the γ-carboxyglutamic acid containing domains of the proteins and

the polar head groups of the phospholipid (for a recent review on the role of phospholipid in blood coagulation, see ref. 19).

The purpose of the experiments described in this chapter is to examine the role of the various components of the intrinsic factor X activating complex by an analysis of the kinetics of factor X activation. In the past, a direct study of this reaction has been difficult for a number of reasons. Methods for purification of the proteins involved were not available and factor X activation could only be followed by a coagulation assay. Such an assay is, however, only possible in the presence of other coagulation factors and may be influenced by a number of feedback reactions.

A more detailed study of the activation reaction is now possible because methods are available to purify the proteins involved and factor X_a formation can be measured directly either with the chromogenic substrate S 2222 **) (20) or through the release of an acid soluble radiolabelled activation peptide (21). Fujikawa et al. (1) have shown that the activation of factor X is the result of the cleavage of the same arginyl-isoleucine peptide bond in the heavy chain of factor X both with factor IX_a and factor VIII or tissue factor and factor VII_a as activator. Suomela and Blombäck (20) and Hultin and Nemerson (22) have shown that factor X is activated at a very low rate by factor IX_a in the presence of phospholipid and calcium ions. Addition of thrombin-activated factor VIII increased the rate of factor X_a formation more than 500-fold (22).

Brown et al. (23) reported a K_m for factor X of about 0.2 µM when factor X is activated by human factor IX_a and bovine factor VIII in the presence of calcium ions and phospholipid. No activation of factor X by factor IX_a in the absence of accessory components has been reported in the literature yet.

In this chapter we present experiments designed to study the kinetics of intrinsic factor X activation. The approach is similar to that in chapter 4. Using purified clotting factors and an assay for factor X_a with chromogenic substrate, we are able to show factor X activation by factor IX_a alone and derive kinetic parameters (K_m for factor X and V_{max} for factor X_a formation) for this reaction. We determined the effects of phospholipid and factor VIII on these kinetic parameters. The results of this study allow a first attempt to explain the role of phospholipid and factor $VIII_a$ in the mechanism of factor X activation and appreciating the contribution of the

intrinsic pathway to thrombin formation in vivo.

EXPERIMENTAL PROCEDURES

MATERIALS

S 2222 and S 2238 were purchased from AB Kabi Diagnostica, Stockholm, Sweden. p-NPGB was from Nutritional Biochemicals. Russell's Viper venom, heparin sodium salt (grade I, 170 USP units/mg), STI and ovalbumin were obtained from Sigma. DEAE-Sephadex A-50, Sephadex G-100 and G-25, Sepharose 4B and 6B and CNBr-activated Sepharose 4B were from Pharmacia. AcA 44 was purchased from LKB, Stockholm, Sweden. Trasylol was from Bayer, Leverkusen, Germany. Kaolin light was from BDH Chemicals Ltd. STI was coupled to CNBr-activated Sepharose 4B following the method of Cuatrecasas (24). Heparin was coupled to Sepharose 4B according to the method of Cuatrecasas (24) as described by Fujikawa et al. (25). All reagents used were of the highest grade commercially available.

METHODS

Proteins

Bovine factor IX was purified as described by Fujikawa et al. (25). Bovine factor X_1 and X_2 were prepared according to Fujikawa et al. (26). The factor IX, X_1 and X_2 preparations were homogeneous and pure as determined by gel electrophoresis in the presence of sodium dodecyl sulfate. The specific activities of these preparations, as determined with a clotting assay, were equal to those reported (25,26).

Partially purified contact product was prepared according to Nossel (27) and further purified by heparin-agarose affinity chromatography as suggested by Østerud et al. (28). Contact product was applied to the heparin-agarose column (1.5 x 15 cm) in a buffer containing 0.05 M sodium acetate and 0.3 M NaCl at pH 5.5 and was eluted with a linear gradient of 0.3 to 1.0 M NaCl in 0.05 M sodiumacetate at pH 5.5.

RVV-X was purified from the crude venom as described by Schiffman et al. (29).

Bovine factor X_a was prepared from bovine factor X_2 using RVV-X according to the method of Fujikawa et al. (30).

Bovine factor IX_a was prepared by incubating factor IX (2 mg/ml) at 37°C with the purified contact product (38 µg/ml) in a buffer containing 50 mM Tris-HCl, 50 mM NaCl at pH 8.5 in the presence

of 10 mM $CaCl_2$ (31). After 60 min incubation EDTA and benzamidine were added to result in final concentrations of 15 mM and 20 mM, respectively. The reaction mixture was then applied to a column of DEAE-Sephadex A-50 (1.5 x 30 cm) in 50 mM Tris-HCl, 50 mM NaCl and 20 mM benzamidine at pH 7.9. Factor IX_a was eluted with a linear gradient of 50 mM to 400 mM NaCl (2 x 250 ml) in 50 mM Tris-HCl, 20 mM benzamidine at pH 7.9.

Bovine βfactor X was prepared from factor X_2 by incubation of factor X_2 (3 mg/ml) with factor X_a (36 µg/ml) in the presence of 100 µM phospholipid vesicles (for preparation see below) in a buffer containing 175 mM NaCl, 10 mM $CaCl_2$ and 50 mM Tris-HCl at pH 7.9 at 37°C (cf. ref. 32). Gel electrophoresis in the presence of sodium dodecyl sulfate showed that after 90 min incubation all factor X_2 present was converted into βfactor X and a small amount of βfactor X_a. So after 90 min incubation EDTA was added to a final concentration of 15 mM and the reaction mixture was applied to an AcA 44 column (2.5 x 90 cm) in 0.1 M sodiumcitrate at pH 6.0. βFactor X was eluted with 0.1 M sodium citrate pH 6.0. After pooling, the βfactor X preparation was dialysed against 175 mM NaCl, 50 mM Tris-HCl buffer at pH 7.9 and chromatographed on a STI-Sepharose column (1.5 x 10 cm) to remove factor X_a. βFactor X preparations were homogeneous and pure as determined by gel electrophoresis in the presence of sodium dodecyl sulfate.

Thrombin was purified as a prothrombin activation product as described in chapter 4.

For the preparation of factor VIII, 9 litres of blood were collected in plastic containers containing 1 litre 0.1 M oxalate, 100 mM benzamidine, 20,000 units of heparin and 100,000 KIE units of trasylol. Plasma was obtained by centrifugation of the blood at 0°C for 25 min at 2000 x g in a MSE Mistral 6L centrifuge. $BaSO_4$ (100 mg/ml) was added to the plasma and the suspension was stirred for 20 min at 4°C. The $BaSO_4$ was removed by centrifugation at 0°C for 20 min at 2000 x g. $BaSO_4$ treated plasma was stored at -70°C in 3 litre plastic containers before further use. After thawing and ethanol precipitation as described by Schmer et al. (33), the suspension was centrifuged for 20 min at 2000 x g at -2°C in a MSE Mistral 6L centrifuge. The precipitate containing factor VIII was dissolved in a buffer of 0.017 M barbituric acid, 0.125 M NaCl, 0.05 M 6-aminohexanoic acid at pH 7.0 at room temperature. This was then applied to two coupled Sepharose

6B columns (5 x 100 cm) (34).

Factor VIII was eluted at room temperature with the same barbituric acid buffer containing 0.02% NaN_3 at pH 7.0. After about 950 ml of eluate factor VIII activity appeared well separated from a large protein peak containing fibrinogen. The front of the peak (60 ml) was pooled and the pooled eluate was centrifuged at 190,000 x g for 21 h in a MSE Superspeed 65 ultracentrifuge at 4°C. The pellet (factor VIII) was dissolved in 50 mM Tris-HCl, 175 mM NaCl at pH 7.9 to a concentration of 40 U/ml. Following this procedure the total recovery of factor VIII is about 2%. The specific activity of this preparation, measured with a clotting assay, is about 25 U/mg. The activity is raised 15-fold upon incubation with thrombin. The specific activity of purified factor VIII, measured with a clotting assay is a minimum value, since it is dependent on the dilution of the factor VIII preparation. Higher values were obtained when higher dilutions were tested.

Factor VIII activities were measured in a one-stage coagulation assay in a factor VIII deficient plasma prepared according to Chantarangkul et al. (35). 50 µl buffer containing 0.029 M sodium barbiturate, 0.029 M sodiumacetate, 0.116 M NaCl (pH 7.4), 0.25 mg/ml inosithine and 0.5 mg/ml kaolin light were incubated for 6 min at 37°C with 50 µl of the factor VIII reagent.50 µl of a factor VIII containing sample was added followed by the addition of 50 µl of 0.033 M $CaCl_2$ and the clotting time was measured. Activities of factor VIII were expressed in U/ml assuming 1 U/ml present in normal bovine plasma.

All protein preparations were stored at -70°C after dialysis against 50 mM Tris-HCl, 175 mM NaCl at pH 7.9. Before storage at -70°C, the factor X_1, X_2, βX and IX preparations were passed through a column of STI-Sepharose (0.9 x 20 cm) to reduce the small amounts of factor X_a that might be present in these preparations. No thrombin and factor X_a could be detected in our protein preparations as determined with the chromogenic substrate S 2238 and S 2222.

Protein concentrations

Factor X_a concentrations were determined by active site titration with p-NPGB according to Smith (36). Factor IX_a concentrations were also determined by active site titration with p-NPGB (see chapter 6). Factor X_1, X_2, and βX concentrations were determined after complete activation with RVV-X followed by active site titration with p-NPGB. When protein concentrations are expressed in mg/ml,

they were calculated from the A_{280} using an $A_{280}^{1\%}$ of 14.9 and 14.3 for factors IX and IX_a (31) and of 12.4 for factors X_1, X_2 (37) and ß X.

Phospholipids and phospholipid vesicle preparations

1,2-Dioleoyl-sn-glycero-3-phosphocholine ($18:1_{cis}/18:1_{cis}$-phosphatidylcholine) was prepared by reacylating the cadmium chloride adduct of sn-glycero-3-phosphocholine with the appropriate fatty acyl chloride according to the method of Bear and Buchnea (38). 1,2-Dioleoyl-sn-glycero-3-phosphoserine ($18:1_{cis}/18:1_{cis}$-phosphatidylserine) was prepared from the respective phosphatidylcholine by enzymatic synthesis according to the method of Comfurius and Zwaal (39).

Single bilayer vesicle solutions were prepared according to the method described by De Kruijff et al. (40) by sonication for 10 min in 50 mM Tris-HCl, 175 mM NaCl buffer at pH 7.9 at 0°C (above the phase transition of the lipids used). Sonication was performed using a MSE Mark II 150-Watt ultrasonic disintegrator set at 10 µ peak to peak amplitude. After sonication no pH adjustment was needed. Phospholipid concentrations were determined by phosphate analysis according to Böttcher et al. (41). Vesicle preparations were made of a mixture of 25/75 (mole/mole) of the phosphatidylcholine preparations described above.

Measurement of the rates of factor X_a formation

Activation of factor X by factor IX_a at 37°C either in the presence or absence of phospholipid, $CaCl_2$ and/or factor $VIII_a$ (for further experimental conditions, see legends to the figures) was followed by transferring small aliquots of the reaction mixture after different time intervals to a cuvet (thermostated at 37°C) containing a buffer of 50 mM Tris-HCl, 175 mM NaCl, 0.5 mg/ml ovalbumin and 10 mM EDTA at pH 7.9 in such amounts that the final volume became 2 ml. In the cuvet also 192 µM of the factor X_a specific chromogenic substrate S 2222 is present. Further reaction of factor IX_a with factor X in the cuvet is prevented by dilution and the presence of EDTA. Since factor IX_a has no amidase activity towards S 2222, the absorbance change recorded at 405 - 500 nm on an Aminco DW2 spectrophotometer (set in the dual wavelength mode), is a measure for the amount of factor X_a present in the aliquot.

From a calibration curve made with known amounts of active site

titrated factor X_a, determined under the same assay conditions as described above, the amount of factor X_a present in the aliquot can be calculated. The rate of factor X_a formation in the reaction mixture is calculated from the amounts of factor X_a present in the samples taken at different time intervals.

The amount of factor IX_a present in the incubation mixture was chosen such that less than 2% of the factor X added is converted during the experiment. Rates of factor X_a formation are expressed as mole factor X_a formed per minute per mole factor IX_a present as determined by active site titration.

For the construction of Lineweaver-Burk plots the rates of factor X_a formation were averaged from three independent determinations at each factor X concentration. K_m and V_{max} were determined by statistical analysis of the data as described by Eisenthal and Cornish-Bowden (42) and lines were drawn accordingly.

Gel electrophoresis

Gel electrophoresis in the presence of sodium dodecyl sulfate was carried out as described by Laemmli (43) on gels containing 10% acrylamide, 0.27% N,N^3-methylbisacrylamide and 0.1% sodium dodecyl sulfate. Before application to the gels, aliquots (10 to 50 µl) of the protein preparations or incubation mixtures were added to 50 µl of 2% sodium dodecyl sulfate, 50 mM EDTA (pH 7.9) and kept for 4 min in a boiling water bath. Five percent mercaptoethanol was present in disulfide reduced samples.

RESULTS

The kinetics of factor X activation in the absence of phospholipid and factor VIII

Although there are no reports in literature which demonstrate that factor IX_a alone is able to activate factor X, our kinetic experiments on prothrombin activation in solution (see chapter 4), which showed a high K_m for the substrate prothrombin and a low V_{max}, made clear that it might be possible to activate factor X in solution at high factor X and factor IX_a concentrations. Fig. 1 shows that factor IX_a is indeed able to activate factor X in the absence of calcium ions, factor VIII and phospholipid. Calcium ions stimulate the activation about 7-fold and there is a rather broad optimum around 10 mM Ca^{2+}. The Ca^{2+}-titration curves are independent of the amounts of factor IX_a and X present. Pretreatment of factor IX_a

with 10 mM diisopropylphosphorofluoridate for 1 hour followed by dialysis did not affect the rate of activation. Since factor IX_a is probably the only serine protease involved in blood coagulation that is not sensitive to diisopropylphosphorofluoridate (5, 44), it is unlikely that factor X activation in the above experiment is accomplished by traces of other serine proteases contaminating our factor IX_a preparations.

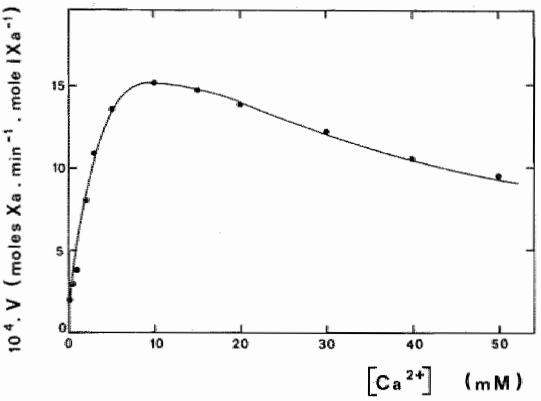


Fig. 1. The Ca^{2+}-dependence of factor X activation by factor IX_a in solution

F X_2 (31.4 µM) was incubated at 37°C with f IX_a (1.1×10^{-3} µmoles/ml) in 200 µl of a buffer containing 50 mM Tris-HCl, 175 mM NaCl, 0.5 mg/ml ovalbumin at pH 7.9 in the presence of varying amounts of $CaCl_2$. Varying amounts of extra NaCl were present to compensate for the changes in ionic strength due to the variation of the $CaCl_2$ concentration. When no $CaCl_2$ was added, the presence of 5 mM EDTA did not influence the rate of f X_a formation. The rate of f X_a formation was calculated from the amounts of f X_a present after 7.5 and 15 min of incubation.

To allow a kinetic analysis of the reaction, it is necessary to demonstrate that the rate of factor X_a formation is constant in time and increases linearly with the amount of factor IX_a present. That this is the case is shown in Fig. 2. We also obtained constant rates of factor X_a formation, proportional to the amount of factor IX_a at higher factor X concentrations and in the absence of calcium ions (data not shown).

The rate of factor X_a formation at various factor X concentrations was determined in the absence of calcium ions and in the presence of 10 mM $CaCl_2$. The data are presented in the form of Lineweaver-

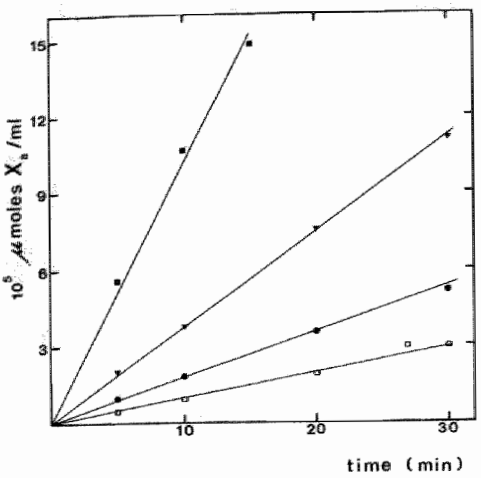


Fig. 2. Time course of factor X activation with different amounts of factor IX_a

F X_2 (31.4 µM) was incubated at 37°C with varying amounts of f IX_a in 200 µl of a buffer containing 50 mM Tris-HCl, 175 mM NaCl, 10 mM $CaCl_2$ and 0.5 mg/ml ovalbumin (pH 7.9). After the time intervals indicated, a sample was taken and assayed for f X_a as described under "Experimental Procedures". The amounts of f IX_a present were: □—□ , 0.55×10^{-3} µmoles/ml; ●—● , 1.1×10^{-3} µmoles/ml; ▲—▲ , 2.2×10^{-3} µmoles/ml; ■—■ , 6.6×10^{-3} µmoles/ml.

Burk plots (Fig. 3A, B). In the absence of calcium ions the K_m for factor X is 299 µM and the V_{max} of factor X_a formation is 2.2×10^{-3} mole $X_a.min^{-1}.mole\ IX_a^{-1}$. In the presence of 10 mM $CaCl_2$ the K_m is 181 µM and the V_{max} is 10.5×10^{-3} mole $X_a.min^{-1}$. mole IX_a^{-1}. The factor X used in these experiments was factor X_2. Hence all subsequent experiments reported in this paper are carried out with factor X_2 as substrate.

The kinetics of factor X activation in the presence of phospholipid and calcium ions

The time course of factor X activation by factor IX_a in the presence of calcium ions and varying factor X and phospholipid concentrations is shown in Fig. 4. To obtain a linear time course of factor X activation, it is essential to preincubate the phospholipid vesicles for 10 min at 37°C in the presence of 50 mM $CaCl_2$. As can be seen, the rate of factor X activation is linear at factor X concentrations

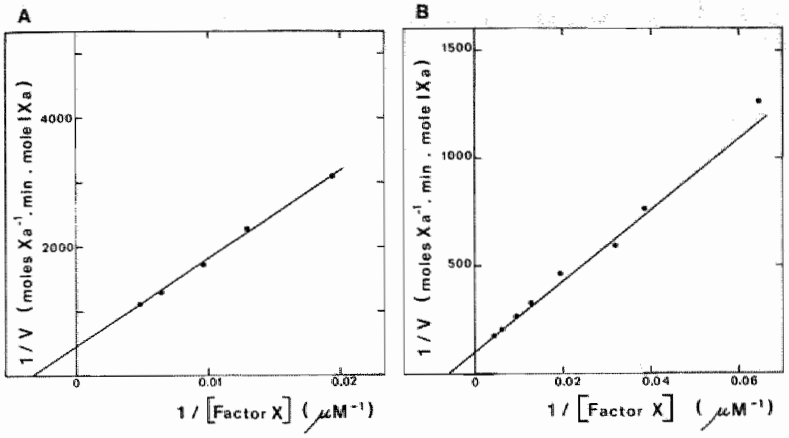


Fig. 3. Lineweaver-Burk plots of factor X activation by factor IX_a in the presence and absence of $CaCl_2$

F X_a formation at varying concentrations of f X_2 was measured at pH 7.9 at 37°C in 200 µl of a reaction mixture containing 50 mM Tris-HCl, 175 mM NaCl, ovalbumin (0.5 mg/ml), f IX_a (1.1×10^{-3} µ moles/ml).

A: in the presence of 5 mm EDTA and B: in the presence of 10 mM $CaCl_2$. The reaction was started by the addition of f IX_a. After 7.5 and 15 min samples were taken and assayed for f X_a as described under "Experimental Procedures". From the amounts of f X_a found, the rate of X_a formation was calculated. The kinetic constants calculated are summarized in Table III.

well below and above the K_m determined at the respective phospholipid concentrations (see below).

Jesty et al. (32, 45) and Fujikawa et al. (46) have shown that factor X_a is able to catalyse a number of feedback reactions on the substrate factor X, especially in the presence of phospholipid and calcium ions. Factor X_a can cleave a small glycopeptide from the carboxyterminal end of the heavy chain of factor X, giving rise to so-called βfactor X. Factor X_a is also able to convert both factor X and βfactor X_a by hydrolysing a specific peptide bond in the aminoterminal region of the heavy chain. Especially, this second feedback reaction may interfere with the rate determination of factor X activation by factor IX_a. It is possible, however, to select experimental conditions such that the feedback factor X

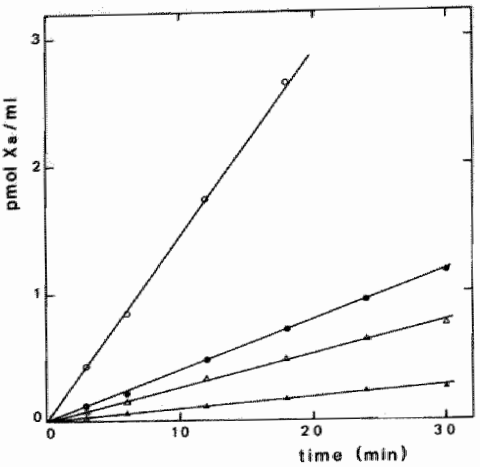


Fig. 4. Time course of factor X activation by factor IX_a in the presence of phospholipid and $CaCl_2$

PL vesicles were incubated 10 min at 37°C in a buffer containing 50 mM Tris-HCl, 175 mM NaCl and 50 mM $CaCl_2$ (pH 7.9). 0.15 ml of this PL suspension was added to 1.2 ml of a reaction mixture containing 50 mM Tris-HCl, 175 mM NaCl, 0.5 mg ovalbumin/ml and f X (amounts indicated below) at pH 7.9 and 37°C. After 3 min, f X activation was started by the addition of 0.15 ml f IX_a (0.067 µM). The final reaction mixture contained: 50 mM Tris-HCl, 175 mM NaCl, 10 mM $CaCl_2$, 0.45 mg/ml ovalbumin, 6.7×10^{-6} µmoles/ml f IX_a and

▲—▲ , 10 µM PL, 0.02 µM f X;
△—△ , 10 µM PL, 0.1 µM f X;
●—● ,100 µM PL, 0.2 µM f X;
O—O ,100 µM PL, 1.0 µM f X.

After the time intervals indicated in the figures, samples were taken and assayed for f X_a. PL vesicles were prepared as described in the "Experimental Procedures".

activation by factor X_a are negligible compared to the contribution by factor IX_a. The low amounts of factor X_a formed in our experiments (0.05 - 2 pmol/ml) can easily be detected on the Aminco DW2 spectrophotometer. When these amounts of factor X_a are incubated with calcium ions, phospholipid and factor X at the conditions employed in our kinetic experiments, no extra factor X_a formation is detectable. As an extra control an aliquot of the reaction mixture (see legend

to Fig. 4) taken after 30 min was analysed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The only protein that could be seen on the gel was factor X (data not shown) and no βfactor X or factor X_a is detectable. From these control experiments we conclude that the factor X_a formation we measure in our experiments results from the action of factor IX_a on αfactor X.

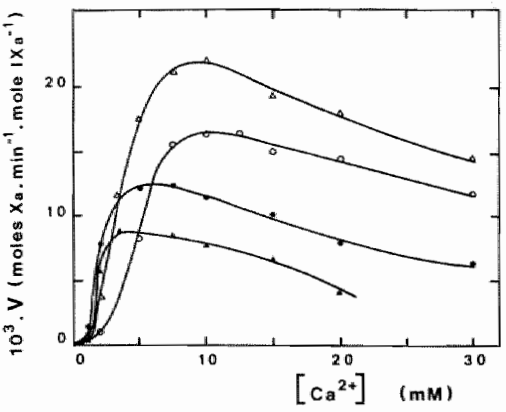


Fig. 5. The Ca^{2+}-dependence of factor X activation by factor IX_a in the presence of phospholipid PL vesicles were incubated at 37°C in 0.3 ml of a buffer containing 50 mM Tris-HCl (pH 7.9), 100 mM NaCl, 0.5 mg/ml ovalbumin and varying amounts of $CaCl_2$. The ionic strength was kept constant by the addition of extra NaCl. After 10 min, 0.95 ml f X (2.0 μM) was added and 4 min later, f X activation was started by the addition of 0.25 ml f IX_a (0.09 μM). Both proteins were dissolved in a buffer containing 50 mM Tris-HCl (pH 7.9),100 mM NaCl and 0.5 mg/ml ovalbumin. The final reaction mixture contained 50 mM Tris-HCl (pH 7.9), 0.5 mg/ml ovalbumin, an amount of NaCl to bring the ionic strength at 0.21, $CaCl_2$ as indicated in the figure, 1.27 μM f X, 1.5×10^{-5} μmoles/ml f IX_a and ▲—▲ , 25 μM PL; ●—● , 50 μM PL; Δ—Δ , 200 μM PL and O—O , 400 μM PL. The rates of f X_a formation were calculated from the amount of f X_a present in the reaction mixture after 7.5 and 15 min. PL vesicles were prepared as described in the "Experimental Procedures".

The Ca^{2+}-dependence of the rate of factor X_a formation at various phospholipid concentrations is shown in Fig. 5. The titration curves are sigmoidal and the optimal Ca^{2+} concentration slightly increases with increasing phospholipid concentrations. The Ca^{2+} optimum is, however, independent of the amounts of factor IX_a and factor X present.

When the factor IX_a concentration is varied at a constant amount of

factor X, calcium ions and phospholipid, the rate of factor X activation observed is directly proportional to the factor IX_a concentration (Fig. 6).

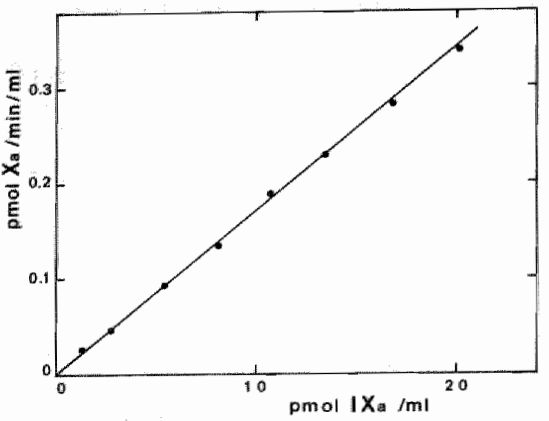


Fig. 6. The effect of factor IX_a on the rate of factor X activation in the presence of phospholipid

PL vesicles were preincubated at 37°C in 50 mM Tris-HCl, 175 mM NaCl, 50 mM $CaCl_2$ at pH 7.9. After 10 min, 50 µl of this suspension was transferred to 300 µl of a buffer containing 50 mM Tris-HCl, 175 mM NaCl, 0.5 mg/ml ovalbumin and 0.833 µM f X at pH 7.9. After 4 min incubation at 37°C, f X activation was started by the addition of 150 µl 50 mM Tris HCl (pH 7.9), 175 mM NaCl buffer containing 0.5 mg/ml ovalbumin and various amounts f IX_a. The final reaction mixture contained 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 10 mM $CaCl_2$, 50 µM phospholipid, 0.5 µM f X, 0.5 mg/ml ovalbumin and amounts of f IX_a indicated in the figure. The rate of f X activation was calculated from the amounts of f X_a present in the reaction mixture after 5 and 10 min. The PL vesicles were prepared as described in the "Experimental Procedures".

The dependence of the rate of factor X_a formation on the factor X concentration is determined at 10 mM $CaCl_2$ and various phospholipid concentrations. A set of Lineweaver-Burk plots is obtained, two of which are shown in Fig. 7. An interesting feature of the Lineweaver-Burk plots shown in Fig. 7 is the hockey-stick shape of the plot. Above a certain factor X concentration, no further increase of the

rate of factor X_a formation is observed. The factor X concentration at which the Lineweaver-Burk plot levels off increases with the phospholipid concentration. A number of explanations is possible for this phenomenon. Since they depend on the model for the mode of action of phospholipid in the factor X activating complex, the explanations will be treated in the discussion. For calculation of the kinetic parameters K_m and V_{max}, we only used the experimental points where the rate of factor X activation is still dependent on the amount of factor X present.

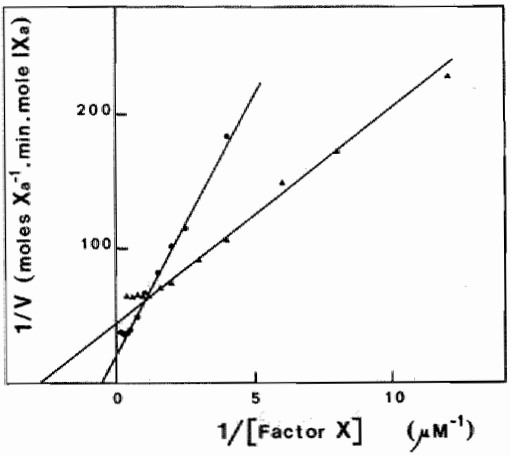


Fig. 7. Lineweaver-Burk plots of factor X activation by factor X_a in the presence of phospholipid

PL vesicles were preincubated at 37°C in a buffer containing 50 mM Tris-HCl, 175 mM NaCl and 50 mM $CaCl_2$ at pH 7.9. After 10 min, 0.1 ml of the PL mixture was transferred to 0.35 ml of a buffer containing 50 mM Tris (pH 7.9), 175 mM NaCl, 0.5 mg/ml ovalbumin and varying amounts of f X. Four min later, f X activation was started by the addition of 0.05 ml f IX_a (0.11 μM). The final reaction mixture contained: 50 mM Tris-HCl, pH 7.9; 175 mM NaCl; 10 mM $CaCl_2$; 0.4 mg/ml ovalbumin; 1.09×10^{-5} μmoles/ml IX_a, varying amounts f X and ▲—▲, 50 μM PL; ●—●, 200 μM PL. The rate of f X_a formation was calculated from the amount of f X_a present in the reaction mixture after 7.5 and 15 min. PL vesicles were prepared as described in the "Experimental Procedures". The kinetic parameters calculated are summarised in Table I.

At 20 μM phospholipid the K_m for factor X is 0.14 μM and the V_{max} of factor X_a formation is 5.8 x 10^{-3} mole X_a.min^{-1}.mole IX_a^{-1}. The rates are expressed per mole of factor IX_a added.

Compared with the results obtained in solution, it is seen that phospholipid lowers the K_m for factor X about 2000-fold with only little effect on the V_{max} of factor X_a formation.

The kinetic constants determined at different phospholipid concentrations are summarized in Table I. There is a gradual increase of the K_m for factor X when the phospholipid concentration is raised.

Table I. Effect of phospholipid on kinetic parameters of factor X activation

Phospholipid (μM)	K_m^{app} (μM)	V_{max} (mole X_a.min^{-1}.mole IX_a^{-1})
10	0.058	0.00247
20	0.139	0.00579
50	0.363	0.0226
75	0.409	0.0219
100	0.525	0.0231
150	0.822	0.0295
200	1.83	0.0474
300	1.76	0.0437

The V_{max} of factor X_a formation also increases with the phospholipid concentration. The variation of V_{max} may be due to the fact that the V_{max} is calculated from the amount of factor IX_a added, while it is actually the amount of factor IX_a bound to the phospholipid surface at each phospholipid concentration that determines the rate of factor X activation. The kinetic parameters of factor X activation in solution are such that free factor IX_a does not contribute to factor X_a formation. Therefore, a variation of the amount of factor IX_a bound due to the variation of the phospholipid concentration will then be reflected in the observed value of V_{max}. In that case extrapolation of a double reciprocal plot ($1/V_{max}$ versus $1/[\text{phospholipid}]$) at constant factor IX_a to infinite phospholipid concentration yields the V_{max} for bound factor IX_a (Fig. 8). The V_{max} calculated from this plot is 0.08 mole X_a.min^{-1}.mole IX_a^{-1} bound.

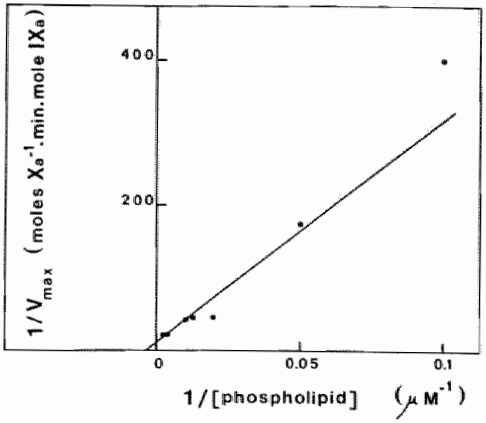


Fig. 8. Double reciprocal plot of V_{max} as a function of the phospholipid concentration

The f X activating mixture consisted of f IX_a, $CaCl_2$ and PL. This plot contains data summarised in Table I.

The kinetics of factor X activation in the presence of phospholipid and factor $VIII_a$

From many reports in the literature, it is obvious that factor VIII participates in the factor X activating complex in an activated form (15, 18, 22). The activity of factor VIII in the intrinsic factor X activation is considerably enhanced by prior incubation with trace amounts of thrombin. The effect of thrombin on the activity of factor VIII is evaluated in general with a clotting assay. Feedback reactions by thrombin and other serine proteases accumulating during clotting hamper a study of the quantitative aspect of this activation. Therefore, we aimed to set up a system in which the activation of factor VIII by thrombin can be followed directly by measuring its effect on factor X activation.

The experimental set-up is as follows. Factor VIII is incubated with varying amounts of thrombin and the time course of activation is followed by transferring after different time intervals aliquots of this activation mixture to a reaction mixture containing a small amount of factor IX_a (2000 x lower than in the experiments of the previous section), factor X, $CaCl_2$ and phospholipid. From the amount of factor X_a formed after 2, 3, and 4 min in this mixture, the rate of factor X_a formation is calculated. This rate is taken as a measure for the amount of factor $VIII_a$ present in the aliquot.

The time course of factor VIII activation by various amounts of thrombin was measured with this assay and is shown in Fig. 9.

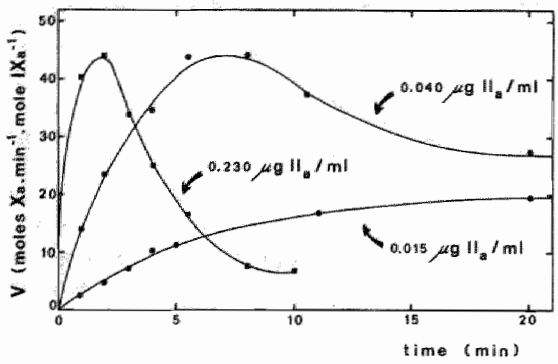


Fig. 9. Time course of activation of factor VIII with various amounts of thrombin

F VIII (10 U/ml) was incubated at 37°C with various amounts of thrombin in a buffer containing 50 mM Tris-HCl, 175 mM NaCl at pH 7.9. The amounts of thrombin present are indicated in the figure. After the time intervals indicated, 0.1 ml of this incubation mixture was transferred to 0.9 ml of a reaction mixture containing all further components required for f X activation. The final concentrations in the reaction mixture (1 ml) were: 50 mM Tris-HCl (pH 7.9); 175 mM NaCl; 10 mM $CaCl_2$; 0.5 mg/ml ovalbumin; f IX_a (0.34×10^{-8} µmoles/ml); 0.015 µM f X and 12.5 µM PL. The PL vesicles were preincubated for 10 min at 37°C in a buffer containing 50 mM Tris-HCl, 175 mM NaCl and 50 mM $CaCl_2$ before addition to the reaction mixture. Two, three and four min after the addition of f $VIII_a$, aliquots from the reaction mixture were assayed for f X_a. The rate of f X_a formation was calculated from the amounts of f X_a present in these aliquots. PL vesicles were prepared as described under "Experimental Procedures".

Incubation of factor VIII with thrombin results in a rapid increase of factor $VIII_a$ activity followed by a decrease. Both the rates of activation and inactivation increase with the thrombin concentration. When either factor VIII or thrombin is omitted from the activation mixture no factor X_a is formed in the reaction mixture. This experiment stresses again that factor VIII has to be activated before it can exert its stimulating effect on the activation of factor X by factor IX_a.

We were unable to show activation of factor VIII by factor X_a

neither in the presence nor in the absence of phospholipid and calcium ions. This is in contrast with earlier reported findings (16, 18) that factor X_a can activate factor VIII. We have to emphasize, however,that the amount of factor X_a is limited to 0.5 µg/ml since higher amounts of factor X_a cause autocatalytic factor X activation in the assay system. In our further experiments, factor VIII is activated by incubation with 0.04 µg thrombin/ml for 5 min at 37°C.

The time course of factor X activation in the presence of factor $VIII_a$, calcium ions and 5, 10, or 25 µM phospholipid was determined at a low and a high factor X concentration (Fig. 10). Factor $VIII_a$, activated as described above, was added to a mixture of phospholipid (pretreated with $CaCl_2$), $CaCl_2$, factor IX_a and factor X preincubated 3 min at 37°C. A typical time course of factor X activation shows a lag period of 1-2 min, a four minute period with an apparent constant rate of factor X_a formation followed by a decrease of the activation rate. We have been unable to shorten the lag period by changing the preincubation conditions or the order of addition of the components of the factor X activating mixture. The time course of factor X_a formation did not change when extra factor X_a (up to amounts formed in the above experiment) was included in the reaction mixture. At the moment we have no explanation for the observed lag period. The decrease of the rate of factor X_a formation after five minutes is probably caused by inactivation of factor $VIII_a$, since addition of extra factor $VIII_a$ to the reaction mixture restores the ability to activate factor X (data not shown).

In all subsequent experiments, we calculated the rate of factor X_a formation in the presence of factor $VIII_a$ over the four minute time interval during which the rate was constant.

The Ca^{2+}-titration curve of factor X activation by factor IX_a in the presence of 5 and 25 µM phospholipid and factor $VIII_a$ is shown in Fig. 11. The curves are sigmoidal and the optimal Ca^{2+} concentration under these conditions is about 7.5 mM at both phospholipid concentrations.

When the factor IX_a concentration is varied at a constant amount of factor X, $CaCl_2$, phospholipid and factor $VIII_a$, the rate of factor X activation observed is directly proportional to the factor IX_a concentration (Fig. 12).

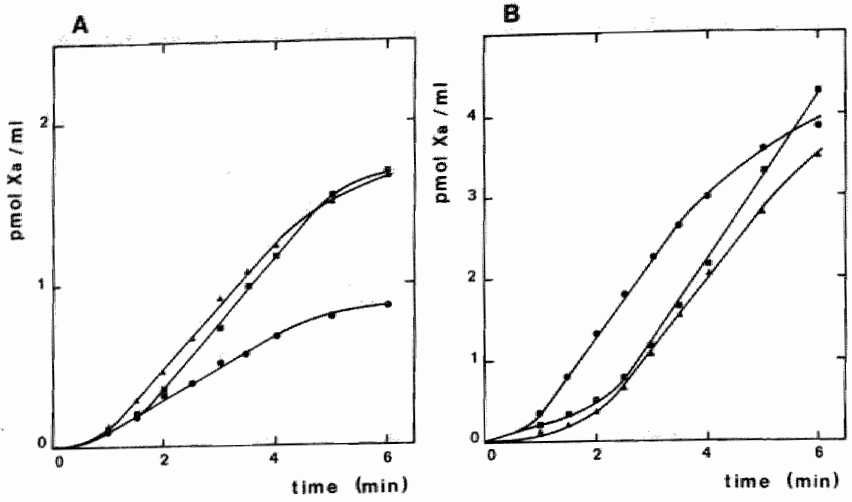


Fig. 10 A, B. Time course of factor X activation by factor IX_a in the presence of phospholipid, factor $VIII_a$ and $CaCl_2$

F VIII (20 U/ml) was activated at 37°C with 0.04 µg/ml thrombin. After 5 min 0.5 ml of the activation mixture was added to 0.5 ml of a reaction mixture containing 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 10 mM $CaCl_2$, 1 mg/ml ovalbumin, 0.68 x 10^{-8} µmoles/ml f IX_a and various amounts of PL and f X at 37°C. Before use, PL vesicles were incubated 10 min at 37°C in a buffer containing 50 mM Tris, 175 mM NaCl and 50 mM $CaCl_2$ at pH 7.9. After the time intervals indicated in the figure, aliquots from this activation mixture were taken and assayed for f X_a. The final reaction mixture contained 50 mM Tris (pH 7.9), 175 mM NaCl, 5 mM $CaCl_2$, 0.5 mg/ml ovalbumin, 0.34 x 10^{-8} µmoles/ml f IX_a, f $VIII_a$ and ●—●, 5 µM PL, 0.005 µM f X; ▲—▲, 10 µM PL, 0.01 µM f X; ■—■, 25 µM PL, 0.025 µM f X (Fig. 10 A) or ●—●, 5 µM PL, 0.05 µM f X; ▲—▲, 10 µM PL, 0.1 µM f X; ■—■, 25 µM PL, 0.25 µM f X (Fig. 10 B). PL vesicles were prepared as described in "Experimental Procedures".

Having established the best experimental conditions for measurement of factor X activation with the complete factor X activating complex, Lineweaver-Burk plots were made at 10 µM phospholipid with various amounts of factor $VIII_a$ present (Fig. 13). The kinetic parameters that are obtained from these plots are summarised in Table II. At high factor $VIII_a$ concentrations the K_m for factor X is 0.063 µM

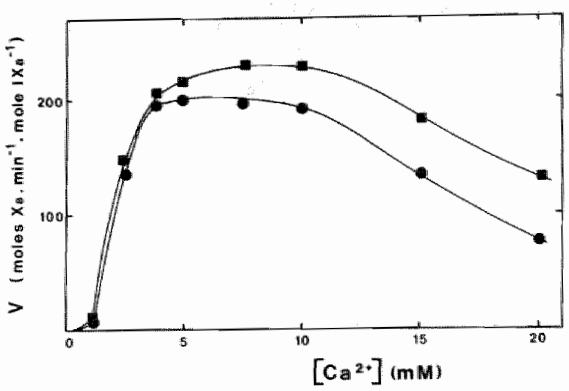


Fig. 11. The Ca^{2+}-dependence of factor X activation by factor IX_a in the presence of phospholipid and factor $VIII_a$

F VIII (20 U/ml) was activated with 0.04 µg/ml thrombin in a buffer containing 50 mM Tris-HCl (pH 7.9) and 175 mM NaCl. After 5 min, 0.5 ml of the activation mixture was added to 0.5 ml of a reaction mixture containing 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 1 mg/ml ovalbumin, 10 or 50 µM PL, 0.68×10^{-8} µmoles/ml f IX_a, 0.04 or 0.2 µM f X and varying amounts of $CaCl_2$. The ionic strength in the reaction mixture was kept constant by the addition of NaCl. Before use the PL vesicles (1 mM) were preincubated for 10 min at 37°C in 50 mM Tris-HCl, 175 mM NaCl, 50 mM $CaCl_2$ at pH 7.9. The incubation mixture with 0 mM $CaCl_2$ contained 5 mM EDTA. Each minute of the incubation, a sample was taken from the reaction mixture and assayed for f X_a. The rate of f X_a formation was calculated from the linear part of the time course of f X_a formation. The final reaction mixture contained $CaCl_2$ as indicated, 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 0.5 mg/ml ovalbumin, 0.34×10^{-8} µmoles/ml f IX_a, f $VIII_a$ and ●—●, 5 µM PL and 0.10 µM f X; ■—■, 25 µM PL, 0.10 µM f X. PL vesicles were prepared as described under "Experimental Procedures".

and the V_{max} is 500 mole $X_a.min^{-1}.mole\ IX_a^{-1}$. When the same experiment was carried out in the absence of factor $VIII_a$, the K_m was 0.058 µM and the V_{max} 0.0025 mole $X_a.min^{-1}.mole\ IX_a^{-1}$ (see Table I). It is obvious that the presence of factor $VIII_a$ hardly affects the K_m for factor X but increases the V_{max} of factor X_a formation about 200,000 fold.

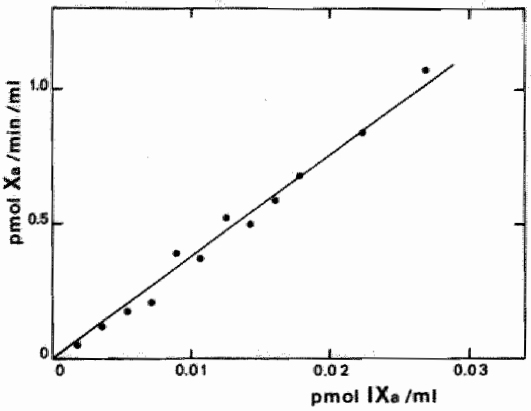


Fig. 12. The effect of factor IX_a on the rate of factor X activation in the presence of factor $VIII_a$ and phospholipid

F VIII (10 U/ml) was activated at 37°C with 0.04 µg/ml thrombin in a buffer containing 50 mM Tris-HCl (pH 7.9) and 175 mM NaCl. After 5 min, 0.1 ml of the activation mixture was added to 0.9 ml of a reaction mixture. The final concentrations in the reaction mixture were: 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 10 mM $CaCl_2$, 0.5 mg/ml ovalbumin, 0.01 µM f X, 10 µM PL, f $VIII_a$ and f IX_a as indicated. Before use, the PL vesicles were preincubated for 10 min at 37°C in 50 mM Tris-HCl, 175 mM NaCl, 50 mM $CaCl_2$ at pH 7.9. After 2, 3, and 4 min, aliquots were taken from the reaction mixtures and were assayed for f X_a. The rate of f X_a formation was calculated from the amount of f X_a present in these aliquots. PL vesicles were prepared as described under "Experimental Procedures".

The V_{max} increases with the amount of factor $VIII_a$ added (Table II). At high concentrations of factor VIII, the rate of activation is not further increases, indicating that it is possible to add saturating amounts of factor $VIII_a$.

In a model in which factor $VIII_a$ is the cofactor of the enzyme factor IX_a this means that at this point factor IX_a is saturated with factor $VIII_a$ and an optimal concentration of factor IX_a - factor $VIII_a$ complex is formed. It is a question, however, whether the model is as simple as that, since the amount of factor $VIII_a$ present also influences the K_m for factor X that is measured (Table II). For this phenomenon we have no obvious explanation yet.

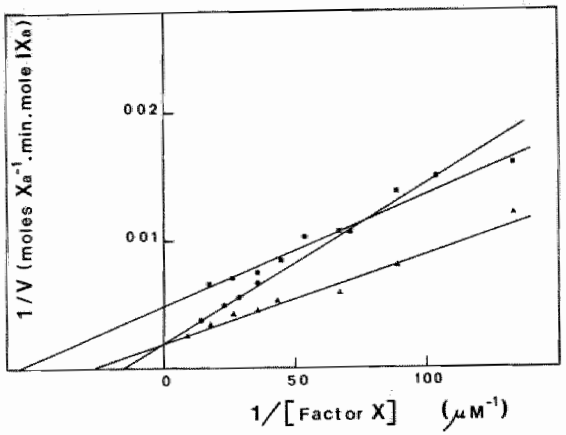


Fig. 13. Lineweaver-Burk plots of factor X_a formation by factor IX_a in the presence of phospholipid and varying amounts of factor VIII

F VIII (20 U/ml) was activated at 37°C with 0.04 µg/ml thrombin in a buffer containing 50 mM Tris-HCl and 175 mM NaCl at pH 7.9. After 5 min incubation varying amounts of this incubation mixture were added to a reaction mixture containing the further components for f X activation in such amounts that the final concentrations in the reaction mixture (1 ml) became: 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 7.5 mM $CaCl_2$, 0.5 mg/ml ovalbumin, 0.34×10^{-8} µmoles/ml f IX_a, 10 µM phospholipid, varying amounts of f X and ■—■, 2 U/ml f VIII, ▲—▲, 8 U/ml f VIII and ●—●, 11 U/ml f VIII. At 2, 3, and 4 min after addition of f VIII to the reaction mixture, aliquots were taken and assayed for f X_a. The rate of f X_a formation was calculated from the amounts of f X_a present in these aliquots. The PL vesicles present in the reaction mixture were prepared as described under "Experimental Procedures" and were incubated for 10 min at 37°C in 50 mM Tris-HCl (pH 7.9) and 175 mM NaCl before addition to the reaction mixture. The kinetic parameters calculated are summarised in Table II.

Lineweaver-Burk plots in the presence of factor $VIII_a$ (8 U/ml) were made at three different phospholipid concentrations (Fig. 14). The kinetic constants obtained from these plots are also summarised in Table II. The K_m for factor X appears to be dependent on the phospholipid concentration to the same extent as observed in the experiments carried out in the absence of factor $VIII_a$ (cf. Table I).

Kinetics of activation of factor X_1 and ßfactor X

Since all experiments reported in this paper were carried out with factor X_2, we were interested whether factor X_1 and ßfactor X behave kinetically identical. Therefore, a number of experiments was re-

Table II. Effect of factor $VIII_a$ and phospholipid on the kinetic parameters of factor X activation

Phospholipid (μM)	$VIII_a$ (Units/ml)	K_m^{app} (μM)	V_{max} (mole X_a.min^{-1}.mole IX_a^{-1})
10	2	0.018	207
10	4	0.022	309
10	8	0.036	497
10	11	0.063	500
5	8	0.024	198
10	8	0.045	439
25	8	0.083	436

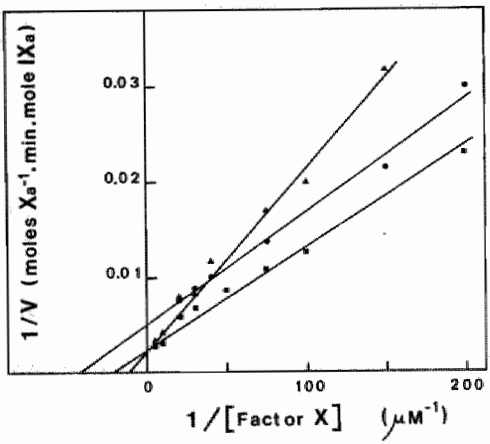


Fig. 14. Lineweaver-Burk plots of factor X_a formation by factor IX_a in the presence of factor $VIII_a$ and varying amounts of phospholipid

The rate of f X_a formation at various f X concentrations was measured at ●—●, 5 μM PL, ■—■, 10 μM PL and ▲—▲, 25 μM PL in the presence of activated f VIII (8 U/ml) and $CaCl_2$ (7.5 mM). For experimental details, see legend to Fig. 13.

peated using factor X_1 and factor βX as substrate. Both factor X_1 and βX showed the same kinetics of activation as factor X_2.

DISCUSSION

Previous studies on the intrinsic activation of factor X suggest that the activator is a complex composed of factor IX_a, factor $VIII_a$, phospholipid and calcium ions (8-11, 22, 23). Several properties of factor IX_a indicate that it is a serine protease and hence will be the enzyme that actually activates factor X. Factor IX_a is highly homologous to other serine proteases involved in blood coagulation (13) and has an esterase activity towards synthetic arginine esters (47). Its enzymatic activity is also inhibited by well-known serine protease inhibitors like antithrombin III (47, 48) and hirudin (49).

When it is accepted that factor IX_a is the enzyme in the factor X-activating complex it is plausible to assume that factor VIII, phospholipid and calcium ions act as cofactors to factor IX_a. In this concept the roles of factor IX_a, factor VIII and phospholipid are analogous to those of respectively factor X_a, factor V_a and phospholipid in the prothrombin-activating complex.

If this model is correct one would expect that factor IX_a can activate factor X in the absence of accessory components. Although no activation of factor X by factor IX_a in solution has been reported yet, Hultin and Nemerson (22) have shown that factor IX_a in the presence of phospholipid and calcium ions, but without factor VIII, slowly activates factor X. The rate of factor X activation was substantially increased when thrombin-activated factor VIII was included in the reaction mixture. A rate enhancement caused by factor VIII has been reported in several other papers (1, 20, 22, 23). These findings extend the analogy with the prothrombin-activating complex. The accessory components phospholipid and factor $VIII_a$ stimulate factor X activation by factor IX_a, like phospholipid and factor V_a increase the rate of conversion of prothrombin to thrombin by factor X_a (50, 51).

In this chapter it is shown that factor IX_a in solution, in the absence of accessory components, is able to catalyse the activation of factor X. This finding supports the concept that factor IX_a is the enzyme in the intrinsic factor X activating complex. It appeared to be possible to carry out a kinetic study and obtain the kinetic parameters (K_m and V_{max}) for the activation reaction in solution. We determined a K_m for factor X of 299 μM and a V_{max} of

2.2×10^{-3} mole $X_a.min^{-1}.mole\ IX_a^{-1}$. The presence of calcium ions has little effect on the kinetic parameters (Table III).

Table III. Effect of the accessory components on kinetic parameters of factor X activation

Composition of factor X activating mixture	K_m^{app} (μM)	V_{max} (mole $X_a.min^{-1}.mole\ IX_a^{-1}$)
IX_a	299	0.0022
IX_a, $CaCl_2$	181	0.0105
IX_a, $CaCl_2$, PL (10 μM)	0.058	0.00247
IX_a, $CaCl_2$, PL (10 μM), $VIII_a$ (11 U/ml)	0.063	500

The fact that we were able to study the kinetics of the reaction in solution made it possible to determine the effect of phospholipid and factor $VIII_a$ on the kinetic parameters of factor X activation. Such a kinetic analysis offers the possibility to gain insight in the role of phospholipid and factor $VIII_a$ in the mechanism of factor X activation. Knowledge of the kinetic parameters is also essential to evaluate the contribution of intrinsic factor X activation to factor X_a formation at physiological conditions.

The kinetic parameters for different factor X activating mixtures are summarised in Table III. Both phospholipid and factor $VIII_a$ cause important changes of the kinetic parameters of factor X activation. In the presence of 10 μM phospholipid, the K_m drops from 181 μM to 0.058 μM, while there is little change of the V_{max}. The effect of factor $VIII_a$ is mainly restricted to the V_{max}. In the presence of factor $VIII_a$ the V_{max} increases about 200,000 fold.

The data in Table III explain why to this day no factor X activation was found by factor IX_a in the absence of accessory components. The kinetic parameters in solution are such that factor X_a formation can only be measured at very high factor X and factor IX_a concentrations and in addition a sensitive assay for factor X_a is required. Factor X activation by factor IX_a in the presence of phospholipid and calcium ions is easier to detect. The K_m for factor X drops to a value in the μmolar range or lower. This explains the findings of Hultin and Nemerson (22) who measured

factor X activation under these conditions. They carried out their experiment at 1.25 μM factor X. With the complete factor X activating complex (factor IX_a, factor $VIII_a$, phospholipid and calcium ions) factor X_a formation is most readily detectable since both kinetic parameters favour factor X activation.

The data presented in this chapter have important implications for the mode of action of phospholipid in the mechanism of intrinsic factor X activation. Phospholipid has little effect on the catalytic activity of factor IX_a. For free factor IX_a a V_{max} of 0.01 mole $X_a.min^{-1}$. mole IX_a^{-1} is measured (Table III) and for phospholipid bound factor IX_a the V_{max} is 0.08 mole $X_a.min^{-1}.mole\ IX_a^{-1}$ (Fig. 8). The K_m for factor X is dramatically decreased in the presence of phospholipid. There is, however, a considerable increase of the K_m at higher phospholipid concentrations, so a K_m measured in the presence of phospholipid has to be regarded as an apparent K_m. In chapter 4 we have shown that in the prothrombinase complex phospholipid causes a marked decrease of the K_m for prothrombin, and the K_m is also raised at increasing phospholipid concentrations. Hence it seems plausible that the mechanistic basis for the mode of action of phospholipid in both complexes is identical. Two models have been proposed to explain the role of phospholipid in prothrombin activation. Because of the similarity between prothrombin and factor X activation with respect to phospholipid involvement, these models are also applicable to factor X activation. In previous papers (chapter 4, ref. 52) we suggested that the enzyme and substrate are bound to the phospholipid surface and that the amount of bound substrate determines the rate of activation. An increased local substrate concentration at or in the vicinity of the phospholipid surface (cf. ref. 53) can explain the large decrease of the K_m observed in the presence of phospholipid. The K_m measured, expressed in terms of added subtrate, is an apparent K_m which increases when increasing amounts of phospholipid are present since at higher phospholipid concentrations more substrate has to be added to attain the local concentration at which the enzyme bound to the surface works at 1/2 V_{max}. A different model is proposed by Nelsestuen (54). He suggests that the enzyme bound at the phospholipid surface is active on the soluble substrate. Phospholipid causes a decrease of the apparent K_m by altering the binding affinity of the active site of the enzyme for its substrate. Although Nelsestuen does not mention in his paper the increase of the apparent K_m at higher

phospholipid concentration, this can also be explained in this model. Binding of the substrate reduces the concentration in solution. At a higher phospholipid concentration more substrate is bound and hence more substrate has to be added to reach a substrate level in solution at which the enzyme functions at 1/2 V_{max}. It will be obvious that both models can qualitatively explain the effect of phospholipid on the K_m for factor X of the intrinsic factor X activator reported in this paper. The flattening of the Lineweaver-Burk plots observed at high factor X concentrations is most easily explained in the model where bound factor X is the substrate. At high factor X concentrations the binding sites for factor X at the phospholipid vesicles become saturated. The addition of extra factor X cannot further increase the bound factor X concentration and consequently there is no further increase in the rate of activation. At higher phospholipid concentration more factor X is required to saturate the binding sites, hence the levelling off occurs at a higher factor X concentration. It is interesting to notice that the break point in the Lineweaver-Burk plots is observed at factor X concentrations about equal to the concentration of binding sites for factor X present on the kind of phospholipid vesicles used in our experiments (0.011 μM binding sites/ μM phospholipid; ref. 55). A qualitative explanation of the above phenomenon in the model where soluble factor X is the substrate is not readily available. In order to determine which of the two models is valid, the exact binding parameters of factor X binding to the phospholipid vesicles under the conditions of our kinetic experiments have to be known. In that case the amounts of bound and free factor X at each individual point of the Lineweaver-Burk plot can be calculated and it can be verified whether the reaction obeys Michaelis-Menten kinetics with bound or soluble substrate.

At the moment experiments are under way in our laboratory in which binding- and kinetic parameters for various phospholipid mixtures are determined at identical conditions with the aim to discriminate between the two models (cf. chapter 7 and 8).

Factor $VIII_a$ enormously stimulates the V_{max} of factor X activation. No activation of factor X can be detected by factor $VIII_a$ alone. Since factor IX_a, even in the absence of accessory components, can catalyse the formation of factor X_a, we conclude that factor $VIII_a$ acts as a cofactor which accelerates an enzymatic reaction which already occurs in its absence. Factor VIII has to be activated with thrombin to accomplish its stimulatory effect on the V_{max}. When

factor $VIII_a$ is replaced by factor VIII in an experiment carried out at a saturating factor $VIII_a$ concentration, the rate of factor X activation is below 1% of that measured with factor $VIII_a$. The remaining activity cannot be explained by the action of factor IX_a alone. Since our unactivated factor VIII preparation may contain small amounts of factor $VIII_a$, we cannot determine whether native factor VIII can support factor X activation. The V_{max} raises when increasing amounts of factor $VIII_a$ are present. At high concentrations of factor $VIII_a$ the V_{max} is not further increased, which in the co-factor model for factor $VIII_a$ would mean that factor IX_a becomes saturated with co-factor. Under these conditions factor IX_a has a proteolytic activity comparable to factor X_a in the prothrombin activating complex (chapter 4, ref. 56), and to factor VII_a in the extrinsic factor X activating complex (21) determined in the presence of saturating amounts of their respective co-factors factor V_a and tissue factor. For the increase of K_m for factor X observed at increasing factor $VIII_a$ concentrations we have no interpretation yet. A more extensive kinetic analysis will be required to explain this phenomenon. The time course of factor X activation in the presence of factor $VIII_a$ showed a lag period of 1-2 min and a levelling off after about 6 min. Such a time course of activation has been reported earlier (22, 23). The reason for the lag period is unclear. It is not due to an extra activation of factor $VIII_a$ by factor IX_a or factor X_a during the time course of the reaction. Preincubation of thrombin-activated factor VIII with factor IX_a or factor X_a in the presence of calcium ions and phospholipid did not cause disappearance of the lag. The slowing down of the reaction after 6 min is likely due to inactivation of factor $VIII_a$ since addition of extra factor $VIII_a$ restores factor X activation. The high molecular weight form of factor VIII is used in the experiments described in this paper. This preparation, generally referred to as factor VIII/von Willebrand factor (factor VIII/vWF) is a complex of low molecular weight factor VIII coagulant activity and high molecular weight von Willebrand platelet aggregating activity. Recently, Vehar and Davie (18) succeeded in obtaining a highly purified factor VIII coagulant activity. It will be interesting to compare the effects of the factor VIII coagulant activity and factor VIII/vWF on the kinetics of factor X activation.

The physiological significance of the alteration of kinetic parameters will be obvious. Intrinsic factor X activation requires

phospholipid because it lowers the K_m for the substrate to within the range of the plasma concentration (0.2 μM). At high phospholipid concentrations the K_m rises, however, above the plasma factor X concentration. It is possible that this phenomenon plays a role in the physiologic regulation of factor X_a formation. Factor $VIII_a$ via its effect on V_{max} increases the rate of factor X activation to a level where sufficient factor X_a is formed to control haemostasis. It is interesting to compare the kinetic parameters that we found for the intrinsic factor X activator with those of the extrinsic factor X activator determined by Silverberg et al. (21). They found a K_m for factor X of 0.34 μM and a V_{max} of 1900 mole $X_a.min^{-1}.mole\ VII_a^{-1}$. Thus the catalytic efficiency, V_{max}/K_m, for both activator systems is about equal. These data will have to be taken into consideration in a discussion about the contribution of the intrinsic and extrinsic factor X activation to factor X_a formation in vivo.

FOOTNOTES

*) The nomenclature of the blood coagulation factors used is that recommended by the Task Force on Nomenclature of Blood Clotting Zymogens and Zymogen Intermediates.

**) The abbreviations used are:
S 2222, N-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-p-nitroanilide hydrochloride; S 2238, H-D-phenylalanyl-L-pipecolyl-L-arginine-p-nitroanilide dihydrochloride; p-NPGB, p- nitrophenyl-p'-guanidinobenzoate hydrochloride; RVV-X, purified factor X activator from Russell's Viper venom; STI, soybean trypsin inhibitor; PL, phospholipid; f X, factor X; f X_a, factor X_a; f IX, factor IX; f IX_a, factor IX_a; f VIII, factor VIII; f $VIII_a$, factor $VIII_a$.

ACKNOWLEDGEMENTS

We would like to thank Mr.J.H.L.Franssen for the preparation of factor VIII, Mrs.G.W.Janssen-Claessen for excellent technical assistance, Dr. Y.Nemerson for kindly providing the computer program for calculation of the kinetic constants, Mrs.J.W.P.Govers-Riemslag for adaptation of the program,and Prof.Dr.R.F.A.Zwaal for stimulating discussions.

REFERENCES

1. Fujikawa, K., Coan, M.H., Legaz, M.E. and Davie, E.W. (1974) *Biochemistry* 13, 5290-5299

2. Jesty, J. and Nemerson, Y. (1974) *J.Biol.Chem.*249, 509-515
3. Davie, E.W. and Fujikawa, K. (1975) *Ann.Rev.Biochem.*44, 799-829
4. MacFarlane, R.G., Biggs, R., Ash, B.J. and Denson, K.W.E. (1964) *Br.J.Haematol.* 10, 530-541
5. Lundblad, R.L. and Davie, E.W. (1964) *Biochemistry* 3, 1720-1725
6. Schiffman, S., Rapaport, S.I. and Chong, M.M.Y. (1966) *Proc.Soc. Exp.Biol.Med.* 123, 736-744
7. Hougie, C., Denson, K.W.E. and Biggs, R. (1967) *Thrombos.Diathes. haemorrh.* 18, 211-222
8. Barton, P.G. (1967) *Nature* 215, 1508-1509
9. Hemker,H.C.and Kahn, M.J.P. (1967) *Nature* 215, 1201-1202
10. Østerud, B. and Rapaport, S.I. (1970) *Biochemistry* 9, 1844-1861
11. Varadi, K. and Hemker, H.C. (1976)*Thrombos.Res.* 8, 303-317
12. Bucher, K., Nebelin, E., Thomsen, J. and Stenflo, J. (1976) *FEBS Letters* 68, 293-296
13. Katayama, K., Ericsson, L.H., Enfield, D.L., Walsh, K.A., Neurath, H., Davie, E.W. and Titani, K. (1979) *Proc.Natl.Acad.Sci. USA* 79, 4990-4994
14. Hemker, H.C., Kahn, M.J.P. and Devilee, P.P. (1970) *Thrombos. Diathes.haemorrh.* 24, 214-223
15. Østerud, B., Rapaport, S.I., Schiffman, S. and Chong, M.M.Y. (1971) *Br.J.Haematol.*21, 643-660
16. Davie, E.W., Fujikawa, K., Legaz, M.E. and Kato, H. (1975) *Cold Spring Harbor Conference on Cell Proliferation* 2, 65-72
17. Vehar, G.A. and Davie, E.W. (1977)*Science* 197, 374-376
18. Vehar, G.A. and Davie, E.W. (1980)*Biochemistry* 19,401-410
19. Zwaal, R.F.A. (1978) *Biochim.Biophys.Acta* 515, 163-205
20. Suomela, H. and Blombäck, B. (1977) *Thrombos.Res.* 1, 267-281
21. Silverberg, S.A., Nemerson, Y.and Zur, M. (1977) *J.Biol.Chem.* 252, 8481-8488
22. Hultin, M.B. and Nemerson, Y. (1978) *Blood* 52, 928-940
23. Brown, J.E., Baugh, R.F. and Hougie, C. (1978) *Thrombos.Res.* 13, 893-900
24. Cuatrecasas, P. (1970)*J.Biol.Chem.*245, 3059-3065
25. Fujikawa, K., Thompson, A.R., Legaz, M.E., Meyer, R.G. and Davie, E.W. (1973) *Biochemistry* 12, 4938-4945
26. Fujikawa, K., Legaz, M.E. and Davie, E.W. (1972) *Biochemistry* 11, 4882-4891
27. Nossel, H.L. (1964) The contact phase of blood coagulation (Blackwell Scientific Publication, Oxford)

28. Østerud, B. and Rapaport, S.I. (1977) *Proc.Natl.Acad.Sci. USA* 74, 5260-5264
29. Schiffman, S., Theodor, I. and Rapaport, S.I. (1969) *Biochemistry* 8, 1397-1405
30. Fujikawa, K., Legaz, M.E. and Davie, E.W. (1972) *Biochemistry* 11, 4892-4899
31. Fujikawa, K., Legaz, M.E., Kato, H. and Davie, E.W. (1974) *Biochemistry* 13, 4508-4516
32. Jesty, J., Spencer, A.K. and Nemerson, Y. (1974) *J.Biol.Chem.* 249, 5614-5622
33. Schmer, G., Kirby, E.P., Teller, D.C. and Davie, E.W. (1972) *J.Biol.Chem.* 247, 2512-2521
34. Van Mourik, J.A. and Mochtar, I.A. (1970) *Biochim.Biophys.Acta* 221, 677-679
35. Chantarangkul, V., Ingram, G.I.C., Thorn, M.B. and Darby, S.C. (1978) *Br.J.Haematol.* 40, 471-488
36. Smith, R.L. (1973) *J.Biol.Chem.*248, 2418-2423
37. Jackson, C.M., Johnson, T.F. and Hanahan, D.J. (1968) *Biochemistry* 7, 4492-4505
38. Bear, E. and Buchnea, D. (1959) *Can. J. Biochem. Physiol.* 37, 953-959
39. Comfurius, P. and Zwaal, R.F.A. (1977) *Biochim.Biophys.Acta* 488, 36-42
40. De Kruijff, B., Cullis, P.R. and Radda, G.K. (1975) *Biochim.Biophys. Acta* 406, 6-20
41. Böttcher, C.J.F., van Gent, C.M. and Pries, C. (1961) *Anal. Chim.Acta* 24, 203-207
42. Eisenthal, R. and Cornish-Bowden, A. (1974) *Biochem.J.* 139, 715-720
43. Laemmli, U.K. (1970) *Nature* 227, 680-685
44. Aronson, D.L. (1974) *Thrombos.Diathes.haemorrh.* 559, 11-26
45. Jesty, J., Spencer, A.K. and Nemerson, Y. (1974) *J.Biol.Chem.* 249, 5614-5622
46. Fujikawa, K., Titani, K. and Davie, E.W. (1975) *Proc.Natl.Acad. Sci.USA* 72, 3359-3363
47. Kurachi, K., Fujikawa, K., Schmer, G. and Davie, E.W. (1976) *Biochemistry* 15, 373-377
48. Rosenberg, J.S., McKenna, P.W. and Rosenberg, R.D. (1975) *J.Biol.Chem.* 250, 8883-8888

49. Davie, E.W., Fujikawa, K., Kurachi, K. and Kisiel, W. (1979) *Adv.in Enzymol.* 48, 277-318
50. Jobin, F. and Esnouf, M.P. (1967) *Biochem.J.* 102, 666-674
51. Esmon, C.T., Owen, W.G. and Jackson, C.M. (1974) *J.Biol.Chem.* 249, 8045-8047
52. Zwaal, R.F.A., Rosing, J., Tans, G., Bevers, E.M. and Hemker, H.C. (1980) In The Regulation of Coagulation (Mann, K.G. and Taylor, F.B. Eds.) pp. 95-112; Elsevier North Holland Inc, New York
53. Nemerson, Y., Zur, M., Bach, R. and Gentry, R. *ibid.* pp.193-202
54. Nelsestuen, G.L. *ibid.* pp. 31-41
55. Nelsestuen, G.L. and Broderius, M. (1977) *Biochemistry* 16, 4172-4177
56. Nesheim, M.E., Taswell, J.B. and Mann, K.G. (1979) *J.Biol.Chem.* 254, 10952-10962.

CHAPTER 6

THE ACTIVATION OF FACTOR IX BY FACTOR XI_a

Active site titration and development of a spectrophotometric assay

Factor IX is the zymogen form of factor IX_a, a clotting factor that participates in the activation of factor X via the intrinsic pathway. The heavy chain of factor IX_a shows considerable homology with trypsin, trombin and factor X_a which suggests that factor IX_a is a serine esterase which active site is located in the heavy chain (1). Since factor IX_a is capable of activating factor X in the absence of Ca^{2+}, phospholipid and factor VIII, it is likely the enzyme responsible for factor X activation via the intrinsic pathway (see also chapter 5).

In order to be able to quantitate reaction rates of factor X activation by factor IX_a on a molar basis it is necessary to quantitate the amounts of factor IX_a present in the reaction mixture. An active site titration is the most direct method to determine the concentration of active enzymes participating in a given reaction (2). In this chapter it is shown that active site titration of factor IX_a is indeed possible using p-NPGB * as a titrant **.

Factor IX can be activated by factor XI_a and by RVV-X (4, 5; see also chapter 2). Activation by factor XI_a occurs via an intermediate which has no coagulant activity and no esterase activity towards synthetic arginine esters, whereas activated factor IX shows both activities (4,5). Activation of factor IX by RVV-X results in an activated factor IX molecule of the same molecular weight as the zymogen. This so-called meizofactor IX_a has a coagulant and an esterase activity amounting to only half that of the factor IX_a molecule which results from the activation of factor IX by factor XI_a (4).

In this chapter we report experiments in which the time course of activation of factor IX by factor XI_a is monitored in three ways:

1) by measurement of biological activity to which end a spectrophotometric assay was developed using the natural substrate for factor IX_a (i.e. factor X). Activation of factor X by factor IX_a was monitored using the chromogenic substrate S 2222;
2) by active site titration of the active sites generated;
3) by gelelectrophoretic analysis in the presence of sodium dodecyl

sulfate.

It will be shown that the intermediate occurring during activation of factor IX by factor XI_a does not interact with p-NPGB, indicating that the active site is not available.

Theory of titration

When a serine esterase is active site titrated with p-NPGB, a rapid burst of p-nitrophenol is observed followed by a very slow or negligible steady state production of p-nitrophenol. Bender et al. (2,6) have derived kinetic equations for this process according to a three-step mechanism.

$$E + \text{p-NPGB} \underset{}{\overset{K_s}{\rightleftharpoons}} E.\text{NPGB} \xrightarrow{k_2} E - GB + NP \xrightarrow{k_3} E + GB$$

Here E is the enzyme, p-NPGB is the substrate, E.NPGB is the adsorptive enzyme-substrate complex, E-GB is the guanidinobenzoyl enzyme that results from the stoichiometric reaction of enzyme with substrate liberating 1 eq of p-nitrophenol (NP) and GB is the free p-guanidinobenzoate that results from the deacylation of the E-GB complex.

When $[S]_o >> [E]_o$ the following equation describes the production of p-nitrophenol in time:

$$[NP]_t = A.t + \pi(1-e^{-bt}) \qquad (I)$$

This can be recognised to consist of a steady state part (A.t) and of a presteady state part ($\pi.(1-e^{-bt})$). The rate with which the exponential term drops to zero is determined by the operational first order rate constant (b) of the presteady state reaction.
The burst of p-nitrophenol production is proportional to the concentration of the enzyme $[E]_o$ and is given by:

$$\pi = [E]_o \cdot \left\{ \frac{k_2/(k_2+k_3)}{1+K_{m\ app}/[S]_o} \right\}^2 \qquad (II)$$

Here $K_{m\ app} = K_s.k_3/(k_2+k_3)$ (III)

Equation II can be rearranged to give:

$$1/\sqrt{\pi} = \frac{k_2+k_3}{k_2\ \sqrt{[E]_o}} + \frac{(k_2+k_3).K_{m\ app}}{k_2.\ \sqrt{[E]_o}} \cdot \frac{1}{[S]_o} \qquad (IV)$$

When π is measured at different substrate concentrations, a plot of $1/\sqrt{\pi}$ vs $1/[S]_o$ gives a straight line which intercept at the $1/[S]_o = 0$ axis is $(k_2+k_3).k_2^{-1}.[E]_o^{-\frac{1}{2}}$. However, in order to be of practical use, it is desirable that titration of the enzyme can be carried out at one substrate concentration which gives directly $[E]_o$. As can be seen from eq. II, $= [E]_o$ only when $k_2 >> k_3$ and $[S]_o >> K_{m\ app}$. Therefore, k_2, k_3 and $K_{m\ app}$ have to be determined. In practice, $K_{m\ app}$ is very low and cannot be accurately determined from the $1/\sqrt{\pi}$ vs $1/[S]_o$ plot and it is therefore calculated from the values of k_2, k_3 and K_s using eq. III.

k_3 can be determined after isolation of the acylenzyme intermediate by following the first order dissociation. A plot of the logarithm of the fraction of enzyme remaining acylated at time t vs time gives a straight line with slope $-k_3$.

From the presteady state part of the reaction (k_2+k_3) and K_s may be determined. As can be seen from eq. I, the first order rate constant b from the presteady state reaction can be obtained from a plot of the logarithm of the extrapolated steady state line $(A.t + \pi)$ minus the actual amount of p-nitrophenol produced at time t vs time. The equation for b as a function of substrate concentration $[S]_o$ is given by

$$b = \frac{(k_2+k_3).[S]_o + k_3.K_s}{K_s + [S]_o} \qquad (V)$$

When $k_3.K_s << (k_2+k_3)$, a condition usually met, this can be rearranged to

$$1/b = \frac{1}{k_2+k_3} + \frac{K_s}{k_2+k_3} \cdot \frac{1}{[S]_o} \qquad (VI)$$

Thus a plot of $1/b$ vs $1/[S]_o$ gives (k_2+k_3) and K_s.

EXPERIMENTAL PROCEDURES

Materials

S 2222 was purchased from AB Kabi Diagnostica, Stockholm, Sweden. p-NPGB was from Nutritional Biochemicals. Russell's Viper venom, heparin sodium salt (grade I, 170 USP units/mg), STI, egg-yolk phosphatidylcholine and ovalbumin were obtained from Sigma.

DEAE-Sephadex A-50, Sephadex G-25 and G-100 were products of Pharmacia. Agarose (Biogel A-15 M) was obtained from Bio-Rad. All reagents used were of the highest grade commercially available.

METHODS

Proteins

Purified contact product, RVV-X, bovine factor IX, bovine factor X_2, bovine factor IX_a and bovine factor X_a were prepared as described in chapter 5. Before storage at -70°C, the protein preparations were dialysed against a buffer containing 50 mM Tris-HCl, 175 mM NaCl at pH 7.9.

Protein concentrations

Protein concentrations were determined as described in chapter 5.

Phospholipids and phospholipid vesicle preparation

Brain phosphatidylserine was prepared as described by Sanders (7). Single bilayer vesicle solutions of a mixture of brain phosphatidylserine and egg-yolk phosphatidylcholine (25/75 w/w) were prepared according to de Kruijff et al. (8) by sonication for 10 min in 50 mM Tris-HCl, 175 mM NaCl at pH 7.9. Sonication was performed using a MSE Mark II 150 Watt ultrasonic disintegrator set at 10 μ peak to peak amplitude. After sonication no pH adjustment was needed.

Titration of activated factor IX

Titration experiments were conducted at 37°C in thermostated cuvets in an Aminco DW-2 spectrophotometer set in the split beam mode at 405 nm. In a typical experiment the sample cuvet contained 40 μg factor IX_a in 800 μl 0.05 M sodium veronal buffer (pH 8.3) in the presence of 20 mM $CaCl_2$. The reference cuvet contained 800 μl 0.05 M sodium veronal buffer and 20 mM $CaCl_2$. After an appropriate time to allow for temperature equilibration 5 μl of a 0.02 M solution of p-NPGB in dimethylformamide was simultaneously added to the sample and the reference cuvet using matched micropipettes. Under these conditions the presteady state part of the reaction is completed in about 6 minutes.

When factor IX at comparable concentrations is allowed to react with p-NPGB no burst of p-nitrophenol production is observed. However, factor IX gives a low steady state production of p-nitrophenol.

Determination of kinetic constants

k_3 was determined as described by Chase and Shaw (9). 5 µl of 0.1 M p-NPGB in dimethylformamide was added to 1 ml 0.05 M sodium veronal buffer at pH 8.3 containing 350 µg factor IX_a and 20 mM $CaCl_2$. After 30 minutes incubation at 37°C the reaction mixture was applied to a Sephadex G-25 column (1.5 x 10 cm) in 0.05 M sodium veronal, 0.02 M $CaCl_2$ at pH 8.3. After 3 ml of eluate 2.5 ml,containing factor IX_a, was collected and incubated at 37°C. After different time intervals samples were taken and assayed for free factor IX_a as described under assay for activated factor IX. The time at which the reaction mixture had sunk into the column was taken as time zero. The concentration of potentially active enzyme $[E]_o$ was determined by measuring the protein concentration in the eluate according to Lowry et al. and according to Bradford (10,11). Thus the fraction of enzyme remaining acylated can be calculated and a plot of the logarithm of this fraction versus time yields k_3.

k_2 and K_s were determined by analysis of the presteady state part of the reaction at different p-NPGB concentrations. The amount of enzyme not yet acylated ($[E]_o - [E\text{-}GB]_t$) at various time intervals was determined from the difference between the steady state line of p-nitrophenol production (extrapolated back to time zero) and the amount of p-nitrophenol actually produced at time t. From a semilogarithmic plot of the amount of enzyme not yet acylated versus time the operational first order rate constant b of the presteady state reaction is obtained. A plot of 1/b versus $1/[p\text{-NPGB}]_o$ yields then (k_2+k_3) and K_s.

$K_{m\ app}$ can then be calculated from the obtained values of k_2, k_3 and K_s.

Assay for activated factor IX

Since factor X_a formation by factor IX_a in the presence of phospholipid and $CaCl_2$ is linear in time and proportional to the amount of factor IX_a added (see results and chapter 5),this provides an excellent method for measuring the factor IX_a concentration in samples which contain an unknown amount of factor IX_a.

Phospholipid vesicles (250 µM) were incubated in the presence of 50 mM $CaCl_2$ in a buffer containing 50 mM Tris-HCl, 175 mM NaCl at pH 7.9 at 37°C. 100 µl was then transferred to 375 µl of a reaction mixture containing 0.1×10^{-9} mole factor X_2

in the same buffer at 37°C. After 4 minutes incubation factor X activation was started with the addition of 25 µl of a solution of unknown factor IX_a concentration. After 5 minutes 400 µl of the reaction mixture was subsampled to a cuvet (thermostated at 37°C) containing 1600 µl of the same buffer. In the cuvet 192 µM S 2222 was present as well as 15 mM EDTA to block further reaction.

Factor IX_a has no amidase activity towards S 2222. Therefore, the absorbance change recorded at 405 - 500 nm on an Aminco DW-2 spectrophotometer (set in the dual wavelength mode) is a measure of the amount of factor X_a present in the reaction mixture. From a calibration curve made with known amounts of active site titrated factor X_a, determined under the same assay conditions as described above, the amount of factor X_a present in the reaction mixture can be calculated. The rate of factor X_a formation obtained with known amounts of active site titrated factor IX_a, determined also under the same conditions as described above, was used to construct a calibration curve for the amount of factor IX_a present in the reaction mixture.

Gel electrophoretic analysis of factor IX activation

Gel electrophoresis in the presence of sodium dodecyl sulphate was carried out as described by Laemmli (12) with gels containing 10% acrylamide, 0.27% N,N'-methyl bisacrylamide and 0.1% sodium dodecyl sulphate. Aliquots of the activation mixture (10 µl) were diluted 30-fold in 2% sodium dodecyl sulphate and were kept for 3 min in a boiling water bath. 5% Mercaptoethanol was present in disulfide reduced samples. Finally, 50 µl was brought on the gels. In order to get an estimate of the factor IX_a formed and of the factor IX still present in the reaction mixture, gels with known amounts of factor IX and factor IX_a (both in the presence and absence of mercaptoethanol) were run simultaneously. After staining and destaining of the gels according to Fairbanks et al. (13), the gels were scanned on a Gilford Model 250 spectrophotometer.

RESULTS

Assay for activated factor IX

In general, factor IX_a is assayed with a clotting assay in factor IX deficient plasma. However, factor IX deficient plasma is expensive and not readily available. Moreover, although sensitive for factor IX_a, quantitation of factor IX_a in a clotting assay essentially is a

bioassay based on a dose-response curve between the amount of factor IX_a added and the clotting times measured, with all the inherent inaccuracies of such an assay. Therefore, we aimed to devise an assay for factor IX_a in a purified system. Earlier work in our laboratory (cf. chapter 5) suggested that factor IX_a can be quantitated adequately via its ability to activate factor X.

The rate of factor X_a formation by factor IX_a in the presence of 0.2 µM factor X_2, 25 µM phospholipid and 10 mM $CaCl_2$ can be easily measured,is linear in time (cf. chapter 5) and is directly proportional to the amount of factor IX_a present (Fig. 1). The calibration curve for factor IX_a gives a straight line up to concentrations of 1 µg/ml.

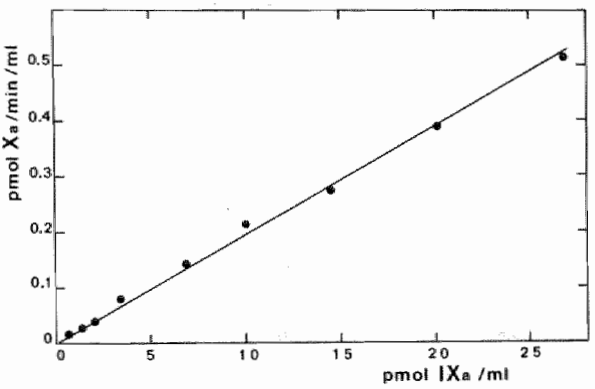


Fig. 1. Calibration curve of the rate of factor X_a formation with varying amounts of factor IX_a

Phospholipid vesicles (125 µM) were preincubated at 37°C in 50 mM Tris-HCl, 175 mM NaCl, 50 mM $CaCl_2$ at pH 7.9. After 10 min, 100 µl of this suspension was transferred to 375 µl of a buffer containing 50 mM Tris-HCl, 175 mM NaCl, 0.5 mg/ml ovalbumin and 0.10 nmol factor X at pH 7.9. After 4 min incubation at 37°C, the reaction was started by the addition of 25 µl of a buffer containing 50 mM Tris-HCl, 175 mM NaCl, 0.5 mg/ml ovalbumin and varying amounts of factor IX_a. The final reaction mixture (0.5 ml) contained 50 mM Tris-HCl, 175 mM NaCl, 0.5 mg/ml ovalbumin, 10 mM $CaCl_2$, 25 µM phospholipid, 0.2 µM factor X and amounts of factor IX_a as indicated in the figure. The rate of factor X activation was calculated from the amounts of factor X_a present in the reaction mixture after 5 min. The phospholipid vesicles were prepared as described under "Experimental Procedures".

Factor IX has no activity in the assay for factor IX_a. Purified contact product has a very low amidase activity towards S 2222 (only detectable when amounts of contact product of 0.5 - 1 mg/ml

are present in the cuvet) and gives no additional factor X_a formation in the assay. However, the amounts of contact product, present in some experiments on factor IX activation (see below) were such that they did not disturb the measurement of factor X_a formation by factor IX_a.

Determination of kinetic constants of factor IX_a for p-NPGB

When factor IX_a is active site titrated with p-NPGB at 37°C in 50 mM veronal at pH 8.3, a burst of p-nitrophenol production is observed followed by a slow steady state p-nitrophenol production (Fig. 2).

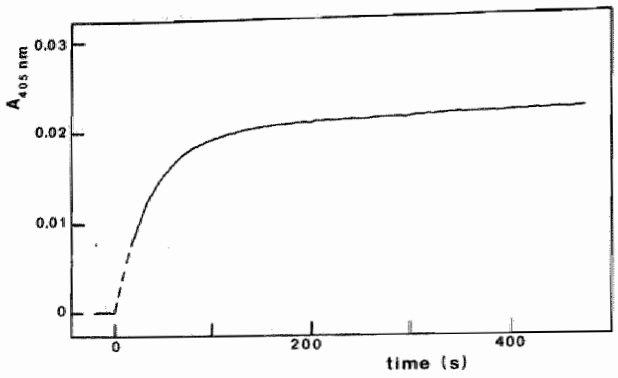


Fig. 2. Titration of 0.8 ml of a solution of factor IX_a at 37°C

Factor IX_a was titrated with p-NPGB at 37°C in 50 mM sodium veronal, 20 mM $CaCl_2$ at pH 8.3 as described under "Experimental Procedures". The A_{405} (π) was determined by extrapolation of the steady state nitrophenol production to time zero. The factor IX_a concentration thus determined was 1.05 μM in the cuvet using an A_{405} of 18,400 for nitrophenol.

The dependence of this burst on p-NPGB concentration is shown in Fig. 3. At 0.1 mM p-NPGB the observed burst is 98% of the maximal attainable burst. Therefore, p-NPGB meets the criterium that active site titration of the enzyme must be possible at one titrant concentration.

From the linearity of 1/b vs $1/[p\text{-NPGB}]_o$ (Fig. 4) it is concluded that the reaction of factor IX_a with p-NPGB can be described in terms of the three step mechanism described by Bender et al. (2,6). From this plot values of 187 μM for K_s and 0.056 S^{-1} for k_2+k_3 are calculated.

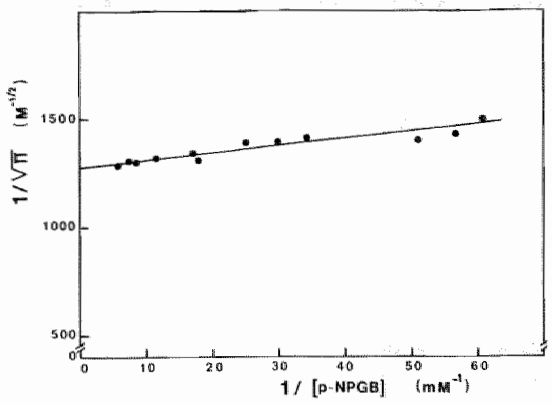
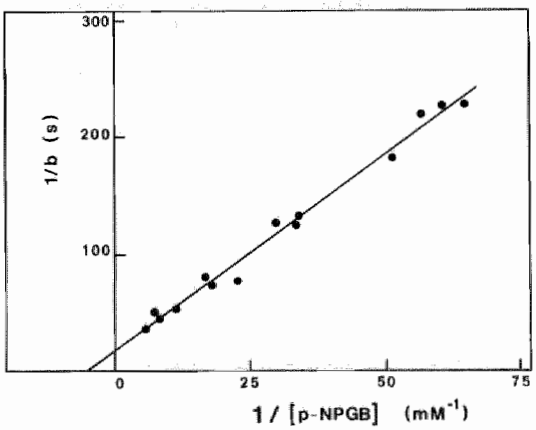


Fig. 3. Dependence of the burst π on p-NPGB concentration

Factor IX_a was titrated at 37°C with varying amounts of p-NPGB in 50 mM sodium veronal, 20 mM $CaCl_2$ at pH 8.3 as described under "Experimental Procedures". The burst π was determined by extrapolation of the steady state nitrophenol production to time zero and factor IX_a concentration was calculated using an A_{405} of 18,400 for nitrophenol. Factor IX_a concentration was 0.62 μM.

Fig. 4. Dependence of the operational first order rate constant (b) of the acylation of factor IX_a on p-NPGB concentration



Factor IX_a was titrated at 37°C with varying amounts of p-NPGB in 50 mM sodium veronal, 20 mM $CaCl_2$ at pH 8.3 as described under "Experimental Procedures". The operational first order rate constant b was determined as described under "Experimental Procedures". The kinetic constants calculated from this plot are summarised in Table I.

Fig. 5 shows that deacylation of the E-GB complex is first order with respect to E-GB. A linear plot for the logarithm of the fraction of the enzyme remaining acylated at time t vs time is obtained and from the slope of this plot a k_3 of 3×10^{-5} S^{-1} is calculated.

The kinetic constants determined are summarised in Table I. These data are in agreement with the data from Byrne et al. (3; see also Table I).

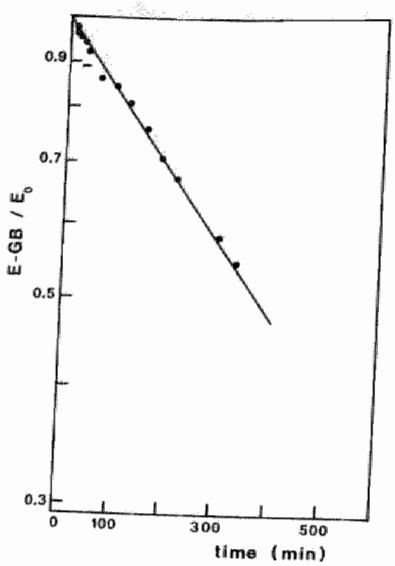


Fig. 5. Deacylation of the factor IX_a-guanidinobenzoate complex

The acylenzyme complex (E-GB) was prepared and isolated as described under "Experimental Procedures". Deacylation of the E-GB complex at 37°C was monitored by measuring the amount of free f IX_a as described under "Experimental Procedures". k_3 was determined as the slope of the line by linear regression. The kinetic constant calculated is summarised in Table I.

Table I. Kinetic constants of factor IX_a and X_a for the reaction with p-NPGB

Enzyme	Temperature	Ca^{2+} (mM)	K_s (μM)	k_2 (s^{-1})	k_3 ($10^5 \times s^{-1}$)	k_2/k_3	$K_{m\ app}$ (μM)
Bovine f IX_a [a]	37°C	20	187	0.056	3.0	1876	0.1
Bovine f IX_a [b]	30°C	10	520	0.13	1.0	13000	0.04
Bovine f X_a [c]	25°C	20	1700	0.085	4.3	2000	5
Bovine f X_a [d]	25°C	0	420	0.19	4.2	4500	0.09

a: this work

b: Byrne et al. (3)

c: Smith (15)

d: Lindhout et al. (16).

Titration of activated factor IX

When different preparations of bovine factor IX_a are active site titrated, the yield is 70 - 95% of the active sites expected, based

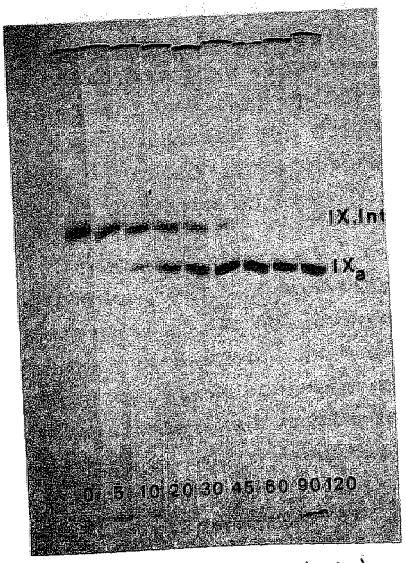
on a protein determination using two different methods (10,11) using a MW of 46,500 (4) and assuming one active site per molecule of factor IX_a. Activation of factor IX with purified contact product of RVV-X yields 95 - 100% of the active sites expected,based on protein determination, using a MW of 55,400 (14) for the zymogen and assuming the presence of one active site per molecule. This confirms the notion that activation of factor IX by factor XI_a or by RVV-X generates one active site per molecule of zymogen.

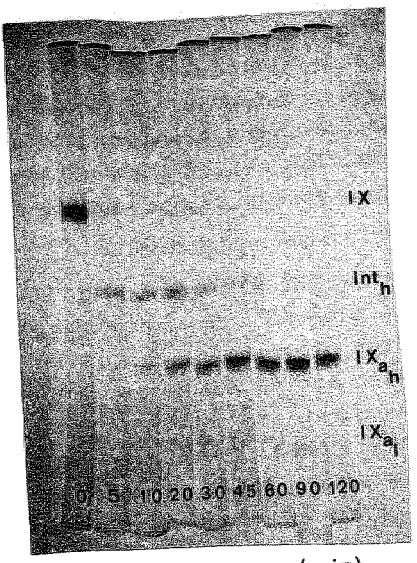
Activation of factor IX

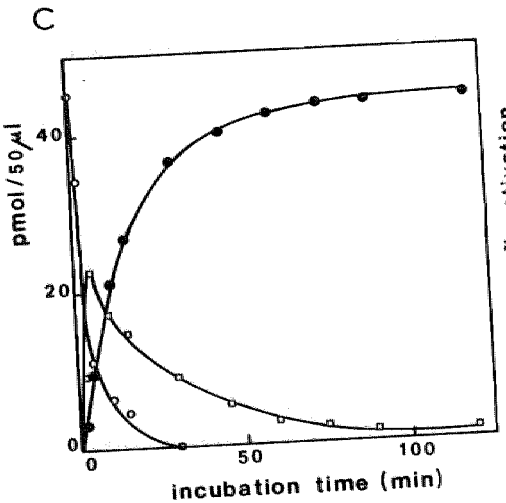
During the activation of factor IX by factor XI_a two peptide bonds are cleaved and an activation peptide of 9,000 MW is liberated (5). After cleavage of the first peptide bond an intermediate is formed which has no coagulant activity and no esterase activity (4,5). Coagulant and esterase activity appear after the cleavage of the second bond. Therefore, we investigated whether the appearance of the active site, as determined by titration with p-NPGB coincided with the cleavage of the second bond. Fig. 6 shows the time course of activation of factor IX as followed by gel electrophoresis, by active site titration and by the activity in the assay for factor IX_a. It can be seen that the appearance of the active sites does not correlate with the disappearance of factor IX. After 10 minutes incubation, 13% of the zymogen is left but only 44% of the active sites has appeared. Therefore, it is concluded that the active site comes free when the second bond is cleaved in the intermediate.

Activation of factor IX by RVV-X results in the formation of so-called meizofactor IX_a. This molecule has the same molecular weight as the zymogen. Meizofactor IX_a was reported to have half of the specific activity of the normal factor IX_a molecule both in a clotting assay and in an esterase assay with tosyl-L-arginine methyl ester (4). This can be due to a difference in kinetic parameters for the substrate of the two different forms of factor IX_a or it can be due to the fact that RVV-X activated factor IX results in the appearance of only half the maximal attainable sites.

Activation of factor IX by RVV-X results in the same number of active sites as is the case when factor IX is activated by contact product (3). Moreover, when contact product was added after activation with RVV-X, meizofactor IX was completely converted into normal factor IX_a and there occurred no change in the number of active sites present (data not shown). Therefore, it is concluded that activation


IX,Int
IX_a
0 5 10 20 30 45 60 90 120
incubation time (min)


IX
Int_h
IX_{a_h}
IX_{a_l}
0 5 10 20 30 45 60 90 120
incubation time (min)


C
pmol/50µl
40
20
0
0
50
100
incubation time (min)

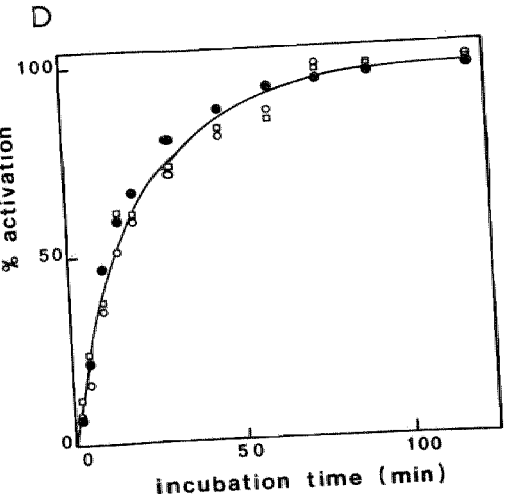

D
% activation
100
50
0
0
50
100
incubation time (min)

Fig. 6. Activation of factor IX by factor XI followed using gel-electrophoresis, active site titration and by measurement of factor IX_a in the spectrophotometric assay

Factor IX (1.5 mg/ml) was activated with purified contact product (30 µg/ml) in a buffer containing 50 mM Tris-HCl, 50 mM NaCl at pH 8.5 at 37°C. After the time intervals indicated samples were taken and active site titrated, assayed for factor IX_a and brought on the gels as described under "Experimental Procedures". Simultaneously, gels were run with samples containing known amounts of factor IX (reduced and unreduced) and factor IX_a (unreduced). After staining and destaining, the gels were scanned on a Gilford Model 250 spectrophotometer.

From scanning of the gels containing the known amounts of factor IX_a (unreduced) a calibration curve was constructed in order to calculate the amounts of factor IX_a present in the unrecuced samples from the reaction mixture.

From scanning of the gels containing the known amounts of factor IX (reduced), a calibration curve was constructed in order to calculate the amount of factor IX present in the sample (reduced) from the reaction mixture.

Assuming that factor IX and the intermediate give the same intensity of staining in the unreduced samples, the amount of factor IX + intermediate present in these samples can be calculated using the calibration curve obtained from scanning of the gels with the known amounts of factor IX (unreduced). Thus the amount of intermediate in the sample can be calculated.

6 A: Gel pattern of activation (unreduced samples)

6 B: Gel pattern of activation (reduced samples)

6 C: Data obtained from gel scanning; ●—● , amount of f IX_a present in the sample; O—O , amount of f IX present in the sample; □—□ , amount of intermediate present in the sample

6 D: Percentage of activation at different time intervals as measured by: ●—● , gel scanning; □—□ , active site titration; O—O , f X_a formation by f IX_a in the spectrophotometric assay.

of factor IX by RVV-X results in an activated factor IX molecule wit its active site free, which has less favourable kinetic parameters for factor X activation and for esterase activity towards tosyl-L-arginine methyl ester.

DISCUSSION

In this chapter it is shown that quantitation of factor IX_a on a molar basis is readily possible by active site titration of the enzyme with p-NPGB. In Table I the kinetic constants of the reactior of factor IX_a with p-NPGB are summarised together with the constants for the reaction of factor X_a with p-NPGB. As can be seen from Table I, the kinetic constants reported here are comparable to those reported by Byrne et al. (3), who determined the kinetic constants in the presence of 10 mM $CaCl_2$ at 30°C. The k_3 that we measure is higher which may be due to the fact that we determined k_3 at 37°C. The k_2 determined by Byrne et al. in the absence of Ca^{2+} is 0.11 S^{-1} whereas k_3 and K_s remain the same. Therefore, it appears that the differences in k_2 and K_s compared with those determined by Byrne et al. are not due to the difference in Ca^{2+} concentration. From the data taken from Smith (15) and from Lind-hout et al. (16) for the reaction of factor X_a with p-NPGB it is clear, however, that differences in Ca^{2+} concentrations may result in considerable changes in k_2 and K_s.

The rate of factor X_a formation in the spectrophotometric assay for factor IX_a is proportional to the amount of active sites present. Different factor IX_a preparations which contain an equal amount of active site give the same rate of factor X_a formation. Factor X_a formation by factor IX_a is completely blocked by the presence of p-NPGB and reappears upon deacylation (see Fig. 5). From these observations we conclude that the site that reacts with p-NPGB is the same site or close to the site which is responsible for the activation of factor X.

The experiments on the activation of factor IX with purified contact product or RVV-X show that both factor IX_a and meizofactor IX_a have the same number of active sites. This is in agreement with the data of Byrne et al. (3). Factor IX_a and meizofactor IX_a were reported to exhibit different specific clotting and esterase activities (4). Since the number of active sites is the same, the kinetic parameters of these two enzymes for the substrate should differ. Byrne et al. showed this indeed to be the case for the

kinetic parameters for benzoyl-L-arginine ethyl ester in the absence of Ca^{2+}. However,in the presence of 10 mM Ca^{2+} the kinetic parameters of the two enzymes are about the same. It will be interesting to compare the kinetic parameters of factor IX_a and meizofactor IX_a for factor X activation to see whether they show any difference.

The intermediate which occurs during the activation of factor IX with contact product has neither coagulant activity nor esterase activity (4). It is shown here that there is neither reaction with p-NPGB. This indicates that the active site is not yet free in this intermediate. This might be due to the fact that the 9,000 MW activation peptide still present in the intermediate prevents the formation of a productive Michaelis complex. However, cleavage of the second bond in the zymogen by RVV-X does result in the formation of an active site pocket which is comparable to that of the normal IX_a molecule in its esterase activity (3). Thus the presence of the 9,000 MW activation peptide in meizofactor IX_a does not seem to be of consequence. Therefore, it is tempting to speculate that the cleavage of the second bond in the factor IX molecule is a prerequisite for the formation of a proper active site pocket. The proof of this, however, will have to await X-ray crystallographic analysis of the molecules involved.

ACKNOWLEDGEMENTS

Part of the chromogenic substrate S 2222 was kindly donated by AB Kabi Diagnostica, Stockholm, Sweden.

FOOTNOTES

Abbreviations used are:
P-NPGB, p-nitrophenyl-p'-guanidinobenzoate; S 2222, N-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-p-nitroanilide hydrochloride; RVV-X, purified factor X activator from Russell's Viper venom; STI, soybean trypsin inhibitor.

REFERENCES

1. Katayama, K., Ericsson, L.H., Enfield, D.L., Walsh, K.A., Neurath, H., Davie, E.W. and Titani, K. (1979) *Proc.Natl.Acad. Sci.USA* 76, 4990-4994
2. Bender, M.L., Begué-Canton, M.L., Blakeley, R.L., Brubacher, L.J., Feder, J., Gunter, C.R., Kézdy, F.J., Killheffer, J.V., Thomas, H.M., Miller, C.G., Roeske, R.W. and Stoops, J.K. (1966)

J.Am.Chem.Soc. 88:24, 5890-5913

3. Byrne, R., Link, R.R. and Castellino, F.J. (1980) *J.Biol.Chem.* 255, 5336-5341
4. Lindquist, P.A., Fujikawa, K. and Davie, E.W. (1978) *J.Biol.Chem.* 253, 1902-1909
5. Fujikawa, K., Legaz, M.E., Kato, H. and Davie, E.W. (1974) *Biochemistry* 13,4508-4516
6. Bender, M.L., Kézdy, F.J. and Wedler, F.G. (1967) *J.Chem.Eduation* 44, 84-88
7. Sanders, H. (1967) *Biochim.Biophys. Acta* 144, 485-487
8. De Kruijff, B., Cullis, P.R. and Radda, G.K. (1975) *Biochim.Biophys. Acta* 406, 6-20
9. Chase Jr, T. and Shaw, E. (1969) *Biochemistry* 8, 2212-2224
10. Lowry, O.H., Rosebrough, N.V., Farr, R.J. and Randall, R.J. (1951) *J.Biol.Chem.* 193, 265-268
11. Bradford, M.M. (1976) *Anal.Biochem.* 72, 248-252
12. Laemmli, U.K. (1970) *Nature* 227, 680-685
13. Fairbanks, G., Stech, T.L. and Wallach, D.F.H. (1971) *Biochemistry* 10, 2606-2617
14. Fujikawa, K., Thompson, A.R., Legaz, M.E., Meyer, R.G. and Davie, E.W. (1973) *Biochemistry* 12, 4938-4945
15. Smith, R.L. (1973) *J.Biol.Chem.* 248, 2418-2423
16. Lindhout, M.J., Kop-Klaassen, B.H.M. and Hemker, H.C. (1978) *Biochim.Biophys.Acta* 533, 342-354.

CHAPTER 7

DETERMINATION OF THE BINDING CONSTANTS OF FACTOR X BINDING TO PHOSPHOLIPID VESICLES

INTRODUCTION

Lipid-protein interactions play an important role in blood coagulation (for a review see ref.1). Factor X activation both via the intrinsic and extrinsic pathway as well as prothrombin activation take place on a phospholipid surface (cf. chapter 2). In order to be able to describe the reactions taking place on the phospholipid surface it is essential to know the binding parameters of the interaction between the phospholipid bilayer and the proteins involved.

A number of methods have been used for the determination of the binding parameters of clotting factors to phospholipid bilayers. Nelsestuen et al. (2,3) carried out an extensive study of factor X and prothrombin binding to phospholipid vesicles of different composition using a light scattering technique.
Bloom et al. (4) used the same technique to determine the binding of factor X, factor V_a and prothrombin to vesicles of a mixture of phosphatidylserine and phosphatidylcholine (25/75;w/w). Since phospholipid vesicles with a mole fraction of phosphatidylserine higher than 30-40% are known to aggregate in the presence of Ca^{2+}-ions (5,6) the light scattering methods are restricted to measurement of binding at low $CaCl_2$ concentrations (less than 5 mM) and to vesicles containing a low mole fraction of phosphatidylserine. This is a serious disadvantage since the optimal Ca^{2+}-concentration for factor X and prothrombin activation in the presence of phospholipid is about 10mM (cf. chapters 4 and 5).
Dombrose at al. (7) used the method of Hummel and Dryer (8) to determine the binding parameters of prothrombin fragment 1 to vesicles of a mixture of phosphatidylglycerol and phosphatidylcholine. This latter method which is also applicable for prothrombin and factor X binding is, however, laborious and requires large amounts of protein.
Nesheim et al. (9) inferred the binding parameters for the components of the prothrombinase complex from the rates of prothrombin activation. This method is, however, subject to the

assumption that the rates of prothrombin activation found are a function of the binding of the different components to the bilayer. It is, however, this latter assumption which one would like to prove. Moreover, the binding constants thus determined will be apparent binding constant dependent on the amount of phospholipid present.

We report here a simple and rapid method for the determination of the binding constants of factor X binding to phospholipid vesicles. This method is based on the finding that RVV-X*) is unable to activate factor X that is bound to phospholipid vesicles, but rapidly activates free factor X. In this chapter it is shown that from the observed rates of factor X_a formation in the presence of phospholipid vesicles the amounts of free and bound factor X can be calculated. This enables determination of the binding parameters of factor X binding to the phospholipid vesicles present in the reaction mixture.

The great advantage of this technique is that the binding of factor X to the vesicles can be measured at the same conditions as used in the kinetic experiments of factor X activation (cf. chapter 5). This is needed in order to be able to interpret the observed kinetics of factor X activation in a model in which factor X bound to the phospholipid surface is the substrate for the enzyme factor IX_a.

Since the method is based on the specific interaction between factor X and RVV-X it is not applicable to other coagulation factors.

EXPERIMENTAL PROCEDURES

MATERIALS

S 2222 was purchased from AB Kabi Diagnostica, Stockholm, Sweden. p-NPGB was from Nutritional Biochemicals. DEAE-Sephadex A-50, Sephadex G-100 and G-200 were products of Pharmacia. STI, ovalbumin and Russel's viper venom were obtained from Sigma. All reagents used were of the highest grade commercially available.

METHODS

Proteins

Bovine factor X, bovine factor X_a and RVV-X were prepared as described in chapter 5. No phospholipase A_2 activity could be detected in the RVV-X preparations. Before storage at - 70 °C the protein preparations were dialysed against a buffer containing 50 mM Tris-HCl, 175 mM NaCl at pH 7.9.

Protein concentrations

Concentrations of factor X and factor X_a were determined as described in chapter 5. RVV-X concentrations were held constant in the experiments at 2.9 ng/ml reaction mixture. The concentration of RVV-X was calculated from the A_{280} assuming an $A_{280}^{1\%}$ of 10.0.

Phospholipid and phospholipid vesicle preparations

were prepared as described in chapter 5.

Determination of the rate of factor X_a formation by RVV-X

Factor X was preincubated at 37 °C in 450 µl of a buffer containing 50 mM Tris-HCl, 175 mM NaCl, 0.5 mg/ml ovalbumin and 11.1 mM $CaCl_2$ at pH 7.9. After 4 minutes, the reaction was started with the addition of 50 µl RVV-X (1.45 ng) in the same buffer. After different time intervals aliquots of the reaction mixture were sampled to a cuvet. The final volume in the cuvet was 2 ml of a buffer containing 50 mM Tris-HCl, 175 mM NaCl, 0,5 mg/ml ovalbumin, 15 mM EDTA and 192 µM S 2222. The EDTA is present to block further reaction. Since RVV-X has no detectable amidase activity towards S 2222, the rate of change in absorbance at 405-500 nm measured on an Aminco DW2 spectrophotometer is a measure for the amount of factor X_a present in the sample. The amount of factor X_a present in the sample was calculated from the measured absorbance change at 405-500 nm using a calibration curve made with known amounts of active site tritated factor X_a under the same conditions as described above. Rates of factor X_a formation are expressed in pmol $X_a.min^{-1}.ml^{-1}$. When phospholipid vesicles were present these were preincubated for 10 min at 37 °C in a buffer containing 50 mM Tris-HCl, 175 mM NaCl and 50 mM $CaCl_2$ at pH 7.9.

The kinetic constants of factor X activation by RVV-X in the absence of phospholipid were obtained from the construction of a Lineweaver-Burk plot using the statistical analysis of the data as described by Eisenthal and Cornish-Bowden (10). For the construction of the Lineweaver-Burk plot the rate of factor X_a formation was averaged from three independent determinations.

RESULTS

The basis of the method to be described is that in a mixture containing free factor X and factor X bound to phospholipid, only free factor X is activated by RVV-X. In order to determine the binding parameters of factor X binding from the rates of factor X_a formation by RVV-X it is necessary to know the kinetics of factor X activation by RVV-X first.

Kinetics of factor X activation by RVV-X in the absence of phospholipid

The reaction of RVV-X with factor X requires $CaCl_2$ (11). Fig. 1 shows the Ca^{2+}-dependence of the reaction at 1 and 0.1 μM factor X.

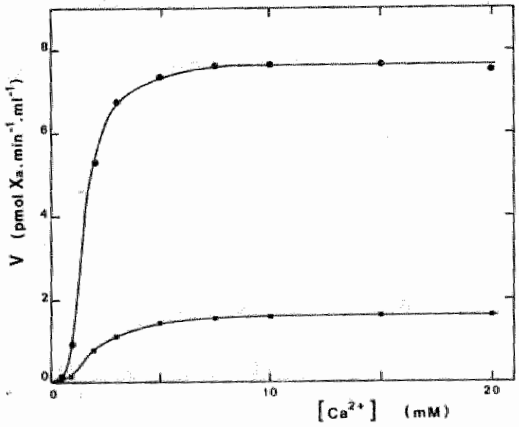


Fig. 1. Ca^{2+}-dependence of f X_a formation by RVV-X at different f X concentrations

RVV-X (1.45 ng) was incubated with factor X at 37 °C in 0.5 ml of a buffer containing 50 mM Tris-HCl, 175 mM NaCl, 0.5 mg/ml ovalbumin and amounts of $CaCl_2$ indicated in the figure at pH 7.9. Reaction was started with the addition of RVV-X. The rate of f X_a formation was calculated from the amounts of f X_a present in the reaction mixture after 2.5 and 5 min. The f X concentrations present were: ●-● , 1 μM fX; ■-■ , 0.1 μM fX.

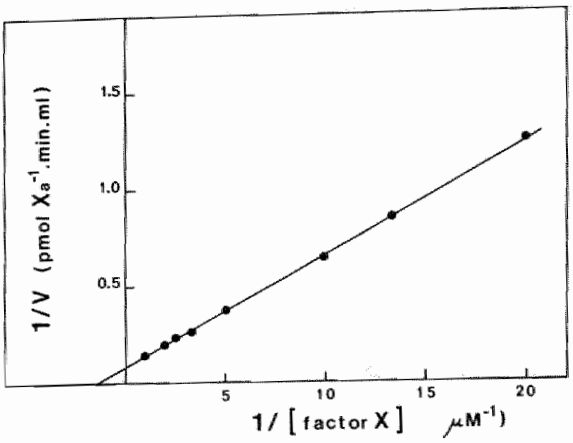


Fig. 2. Lineweaver-Burk plot of f X_a formation by RVV-X

F X_a formation by RVV-X at varying f X concentrations was measured at 37 °C in 0.5 ml of a reaction mixture containing 1.45 ng RVV-X, 50 mM Tris-HCl, 175 mM NaCl, 0.5 mg/ml ovalbumin, 10 mM $CaCl_2$ and varying amounts of f X. The rate of f X_a formation was calculated from the amounts of f X_a present in the reaction mixture after 2.5 and 5 min. The kinetic constants determined are 0.71 µM for the K_m for f X and 4.23 pmol $X_a.min^{-1}.ng^{-1}$ RVV-X present.

These concentrations are well above and below the K_m for factor X (see below). The sigmoidal shape of the curve is due to the presence of the γ-carboxy glutamic acid residues in the factor X molecule (12). Since the rates of factor X_a formation at both factor X concentrations are constant in the range of 5-20 mM $CaCl_2$ we conclude that the kinetic parameters of factor X_a formation are constant in this range of Ca^{2-}-concentrations.
The steady state velocity of the reaction is constant in time and proportional to the amount of enzyme present (data not shown).
The rates of factor X_a formation at varying factor X concentrations were measured at 10 mM $CaCl_2$ and a Lineweaver-Burk plot was constructed (Fig. 2). The kinetic constants determined from this plot are 0.71 µM for the K_m for factor X and a V_{max} of 4.23 pmol X_a. $min^{-1}.ng^{-1}$ RVV-X present. These values are in reasonable aggreement with the kinetic parameters reported by Kosow et al. (11).
Provided that the amount of RVV-X present in a reaction mixture is

known, knowledge of the kinetic parameters of factor X activation enables the determination of an unknown amount of factor X present in the reaction mixture from the observed rate of factor X_a formation using the following equation:

$$X = \frac{v.K_m}{V_{max} - v} \qquad (I)$$

Here X is the unknown concentration of factor X present in the reaction mixture, V_{max} and K_m are the kinetic parameters of factor X activation by RVV-X and v is the observed rate of factor X_a formation.

Inhibition of factor X_a formation by RVV-X in the presence of phospholipid

Fig. 3 shows the time course of factor X activation by RVV-X in the absence and presence of phospholipid vesicles. The steady state velocity of the reaction is markedly decreased by the presence of phospholipid.

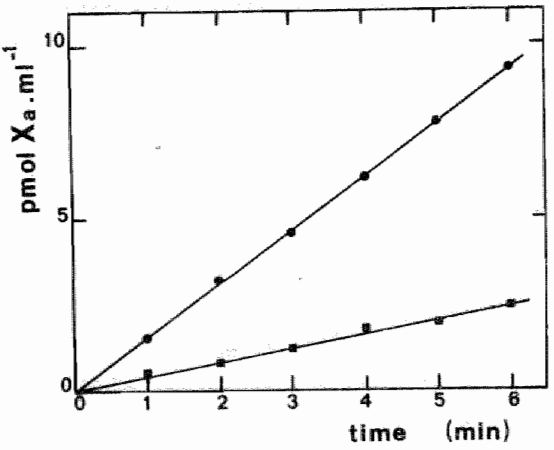


Fig. 3. Time course of f X activation by RVV-X in the presence and absence of phospholipid

F X activation by RVV-X was measured at 37 °C in 1 ml of a reaction mixture containing 50 mM Tris-HCl,175 mM NaCl, 0.5 mg/ml ovalbumin, 10 mM $CaCl_2$, 0.1 µM f X, 2.90 ng RVV-X; ●—●, in the absence of phospholipid; ■—■, in the presence of 50 µM phospholipid vesicles of a mixture of dioleoylphosphatidylserine and dioleoylphosphatidylcholine (1/1;mole/mole). Reaction was started with the addition of RVV-X. Phospholipid vesicles were preincubated for 10 min at 37 °C in 50 mM Tris-HCl, 175 mM NaCl and 50 mM $CaCl_2$ at pH 7.9. After the time intervals indicated samples were taken and assayed for f X_a as described under "Experimental Procedures".

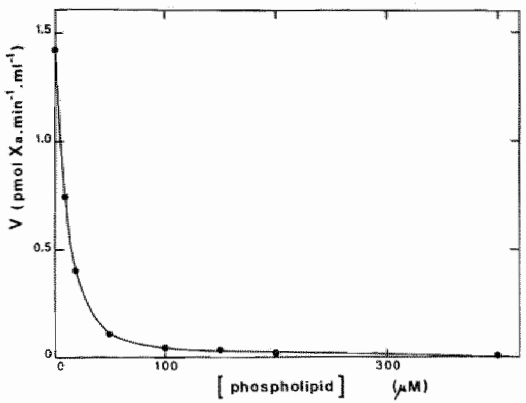


Fig. 4. Effect of phospholipid on the rate of f X_a formation by RVV-X

F X activation by RVV-X was measured at 37 °C in 0.5 ml of a reaction mixture containing 50 mM Tris-HCl, 175 mM NaCl, 0.5 mg/ml ovalbumin, 10 mM $CaCl_2$, 0.1 μM f X, 1.45 ng RVV-X and amounts of phospholipid as indicated in the figure. Reaction was started with the addition of RVV-X. Phospholipid vesicles were preincubated for 10 min at 37 °C in 50 mM Tris-HCl, 175 mM NaCl and 50 mM $CaCl_2$ at pH 7.9. The rate of f X_a formation was calculated from the amounts of f X_a present in the reaction mixture after 2.5 and 5 min.

Fig. 4 shows the dependence of the steady state velocity on the phospholipid concentration. At 400 μM phospholipid the remaining rate of factor X_a formation is less then 1% of the rate measured in the absence of phospholipid. This inhibition by phospholipid can be explained assuming that either RVV-X binds to the phospholipid vesicles and becomes inactive or that RVV-X is not able to activate factor X molecules bound to the phospholipid bilayer. Factor X_a bound to phospholipid has the same activity towards S 2222. Therefore, this explanation can be ruled out.

To distinguish between these two possibilities the following experiment was carried out. Four centrifugation tubes were filled with mixtures of varying composition of RVV-X, factor X and liposomes of a mixture of dioleoylphosphatidylserine and dioleoylphosphatidylcholine (50/50; mole/mole) in a buffer containing 50 mM Tris-HCl, 175 mM CaCl, 0.5 mg/ml ovalbumin and

Table I. Effect of f X binding to liposomes on the rate of factor X_a formation by RVV-X

contents				rate of factor X_a formation (pmol $X_a.min^{-1}.ml^{-1}$)	
	liposomes[a] (μM)	RVV-X (ng/ml)	factor X (μM)	before centrifugation	after centrifugation
tube 1	200	2.9	x	n.d.	6.5[b]
tube 2	x	2.9	x	n.d.	6.6[b]
tube 3	200	x	0.1	0.44[c]	0.37[d]
tube 4	x	x	0.1	3.76[c]	3.75[d]

a) phospholipid liposomes consisted of a mixture of dioleoylphosphatidylserine and dioleoylphosphatidylcholine (50/50; mole/mole)

b) rate of f X_a formation was measured after addition of f X to the supernatant to result in a concentration of 4.2 μM

c) rate of f X_a formation was measured after addition of RVV-X (2.9 ng/ml)

d) rate of f X_a formation was measured after addition of RVV-X (2.9 ng/ml) to the supernatant.

10 mM $CaCl_2$. The composition of the mixtures is indicated in Table I. After centrifugation at 50,000xg for 45 min of tube 1 and tube 2, tube 1 contained a pellet of the phospholipid liposomes. Factor X was added to both supernatants to a final concentration of 4.2 μM and the rate of factor X_a formation was measured. Since the rates of factor X_a formation are the same in both supernatants, we conclude that equal amounts of RVV-X are present and that RVV-X does not bind to the liposomes. Before centrifugation samples (0.6 ml) were taken from tube 3 and tube 4, RVV-X was added and the rate of factor X_a formation was measured. Due to the presence of the liposomes the rate of factor X_a formation was markedly decreased for the sample taken from tube 3. After centrifugation RVV-X was added to 0.6 ml of the supernatant of tube 3 and to 0.6 ml of tube 4 and the rate of factor X_a formation was measured.

Since the rates of factor X_a formation before and after centrifugation are the same it is concluded that it is indeed the amount of free factor X which determines the rate of factor X_a formation by RVV-X. Thus RVV-X can be used to measure the amount of free factor X in reaction mixtures with phospholipid present and this can be used to determine the binding parameters of factor X binding to phospholipid vesicles.

Determination of the parameters of factor X binding to phospholipid vesicles

Since RVV-X is only able to activate factor X molecules not bound to the phospholipid bilayer the observed rates of factor X_a formation in the presence of phospholipid can be used to calculate the concentration of free factor X using equation I. From the calculated amount of factor X_{free} and the known amount of factor X added the amount of factor X_{bound} can be calculated.

When the binding of factor X is measured in this way, care has to be taken that the amount of factor X converted does not influence the binding equilibrium. Therefore, the reaction with RVV-X was allowed to proceed for only 1 minute before a sample was withdrawn from the reaction mixture and was assayed for factor X_a. From the amounts of factor X_a found the rate of factor X_a formation was calculated. The amounts of factor X converted were never more than 2% of the amount of free factor X measured. This can be calculated from the amount of factor X_a found in the sample and the amount of free factor X calculated.

It has been shown that factor X_a can autocatalytically convert factor X to factor X_a in the presence of $CaCl_2$ and phospholipid (13, 14). However, the small amounts of factor X_a formed during the activation by RVV-X were such that no autocatalytical factor X_a formation could be measured. Moreover, gelelectrophoretic analysis of a sample in which these small amounts of factor X_a were formed also showed that no β factor X was formed (data not shown).

The binding of factor X to phospholipid vesicles composed of a 1/1 (mole/mole) mixture of dioleoylphosphatidylserine and dioleoylphosphatidylcholine at three different phospholipid concentrations was determined.

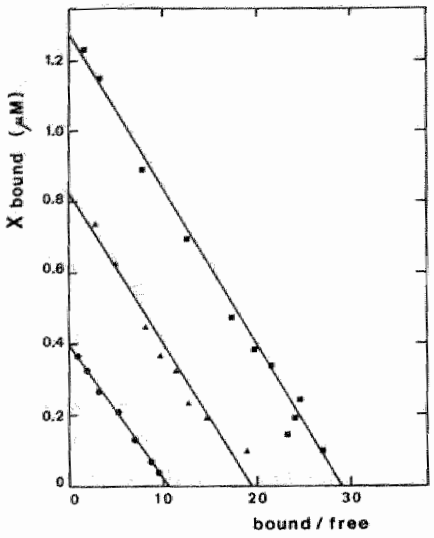


Fig. 5. Scatchard plot of f X binding to phospholipid vesicles.

F X_a formation by RVV-X at different f X concentrations was measured at 37 °C in 0.5 ml of a reaction mixture containing 50 mM Tris-HCl, 175 mM NaCl, 0.5 mg/ml ovalbumin, 10 mM $CaCl_2$, 1.45 ng RVV-X, varying amounts of f X and ●——●, 57 μM PL; ▲——▲, 114 μM PL; ■——■ 171 μM PL.
Reaction was started with the addition of RVV-X. Phospholipid vesicles (1/1; mole/mole; dioleoylphosphatidylserine/dioleoylphosphatidylcholine) were pre-incubated for 10 min at 37 °C in 50 mM Tris-HCl, 175 mM NaCl and 50 mM $CaCl_2$.
The rate of f X_a formation was calculated from the amount of f X_a present in the reaction mixture after 1 min. From the observed rate of f X_a formation the amount of f X_{free} was calculated (see results section).

Fig. 5 shows the Scatchard plots obtained. The binding constants determined are summarised in Table II.
As can be seen the K_d is constant (0.04 μM) and the amount of binding sites found is proportional to the amount of phospholipid present (0.73 μM binding sites per 100 μM phospholipid).

The binding parameters of factor X binding were strongly dependent on the amount of phosphatidylserine present in the vesicles. Fig. 6 shows the dependence of K_d and sites (per 100 μM phospholipid) on the mole fraction of phosphatidylserine present. The binding constants are also summarised in Table II. The K_d and the number of sites depend almost linear on the fraction of phosphatidylserine in the range of 0-30 mole% phosphatidylserine. The K_d decreases from 0.18 μM for vesicles containing 6 mole% phosphatidylserine to 0.03 μM for vesicles containing 37 mole% phosphatidylserine whereas the amount of sites increases from

TABLE II Binding constants for phospholipid vesicles composed of a mixture of dioleoylphosphatidylserine and dioleoylphosphatidylcholine

Total phospholipid concentration	mole fraction phosphatidylserine	K_d	Sites
(μM)	(%)	(μM)	(μM)
57	50	0.038	0.40
114	50	0.043	0.83
171	50	0.044	1.29
100	6	0.176	0.21
100	11.7	0.152	0.42
100	17.3	0.084	0.67
100	23	0.065	0.78
100	37.3	0.032	0.85
100	44	0.030	0.83
100	50	0.017	0.80
100	54	0.019	0.63
100	70	0.048	0.54
100	85	0.046	0.43
100	100	0.07	0.22
100	70[a)]	0.035	0.58
100	85[a)]	0.046	0.43
100	100[a)]	0.05	0.27
100	25[b)]	0.049	0.65
100	100[b)]	0.202	0.29

a) the vesicles used were treated with 10 mM $CaCl_2$ and subsequently 15 mM EDTA was added. After this treatment $CaCl_2$ was added to result in a final concentration of 10 mM;

b) the vesicles used were prepared according to Gerritsen et al (15).

For further experimental details see legends to figures 5 and 6.

0.2 to 0.85 μM respectively.

When the mole fraction of phosphatidylserine is raised the amount of sites is not further increased, in aggreement with the findings of Nelsestuen and Broderius (3). However, the amount of sites present

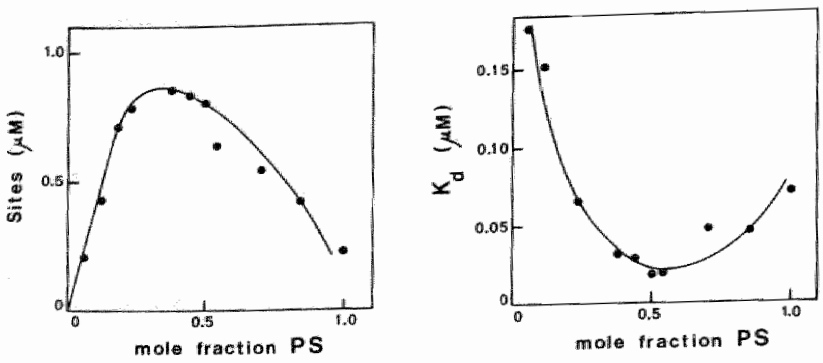


Fig. 6. Effect of the mole fraction of phosphatidylserine present in the vesicles on the parameters of f X binding.

Scatchard plots of f X binding to phospolipid vesicles containing a varying mole fraction phosphatidylserine were obtained by measuring the rate of f X_a formation by RVV-X at varying f X concentrations in the presence of 100 μM PL. For further experimental details see legend to fig. 5. The binding constants determined are summarised in Table II.

drastically decreases for vesicles containing more than 60% phosphatidylserine. For vesicles of pure phosphatidylserine only 0.22 μM sites remain and the K_d has increased to 0.07 μM.

From literature it is known that vesicles composed of brain phosphatidylserine aggregate in the presence of $CaCl_2$ and form structures known as scrolls (5). This can explain the observed decrease in sites since much less surface is then available for the binding of factor X. Addition of EDTA to these scrolls results in the formation of large single bilayer vesicles (5). Therefore, the vesicles we used were first treated with 10 μM $CaCl_2$ and were then treated with 10 mM EDTA. The opaque white suspension becomes clear upon the addition of EDTA. When this process is monitored by measuring the optical density of the phospholipid vesicle solutions at 400 nm it is seen that the large increase in optical density caused by $CaCl_2$ is counteracted by subsequent addition of EDTA. Since the vesicles are now larger the optical density resulting is still higher than that of the sonicated vesicle solution. Subsequent addition of $CaCl_2$,

however, again increases the optical density to the same level as before the addition of EDTA. It was therefore not surprising that treatment of the vesicles in this way did not result in a significant change in the amount of sites present on the phospholipid surface (see table II).

Vesicle solutions of large single bilayer vesicles prepared according to Gerritsen et al. (15) also gave a high increase in optical density upon addition of 10 mM $CaCl_2$ and in this case also the amount of sites found did not significantly change (see table II).

DISCUSSION

To determine binding parameters for factor X binding to phospohlipid vesicles it is necessary to measure either the concentration of free factor X or bound factor X or both. When both the amount of free and bound ligand can be measured accurately over a wide range of concentrations there is almost no limitation as to which K_d can be measured. When only one component is measured there may be limitations as to which K_d's can be measured.

When the concentration of bound ligand is monitored it is in general difficult to determine low K_d's. When K_d is too low the amount of bound ligand is much greater than the amount of free ligand. Therefore, small errors in the measured concentration of bound ligand will produce large errors in the calculated concentration of free ligand.

When the concentration of free ligand is monitored it is difficult to measure high K_d's. In this case almost all of the ligand will be free and small errors in the determined concentration of free ligand will cause large errors in the calculated concentration of bound ligand.

Unfortunately, the kinetics of factor X activation by RVV-X set a strict limit to the highest K_d which can be measured. To allow accurate measurement of X_{free} the highest concentration of X_{free} used (about 4 orders of magnitude above the K_d which has to be determined) must not exceed the K_m for factor X too much. Therefore, since the K_m is 0.71 μM the highest K_d which can be measured is at most 1 μM. The lower limit for the K_d we can determine is experimentally determined by the lowest concentration of factor X_a we can still accurately measure. On the Aminco DW-2 spectrophotometer this is 0.005 nM factor X_a. This concentration should

represent about 1% of the amount of the lowest X_{free} concentration measured. Since this X_{free} concentration will be about $\frac{1}{4}.K_d$ the lowest K_d which can be measured is about 1-2 nM.

The binding parameters obtained for vesicles composed of a mixture of dioleoylphosphatidylserine and dioleoylphosphatidylcholine at varying ratios are summarised in Table II. The K_d and sites we determined at 25 mole% phosphatidylserine are lower than the values reported by Nelsestuen and Broderius (3) and by Bloom et al. (4). These values are summarised in Table III. The experimental conditions we used are, however, significantly different. The lower value of K_d is probably due to the higher $CaCl_2$ concentration since it was reported that K_d is dependent on the amount of $CaCl_2$ present (3). For the difference in sites we have no explanation.

Table III Binding constant for phospholipid vesicles containing 25 mole% phosphatidylserine

Mole fraction phosphatidylserine	$CaCl_2$	K_d	Sites per 100 µM phospholipid
%	mM	µM	µM
23 [a]	10	0.065	0.78
25 [b]	2	0.2	1.5
25 [c]	2	2.45	1.96

a) this work

b) calculated from Nelsestuen and Broderius (3)

c) calculated from Bloom et al. (4)

It was observed that the amount of sites present was drastically decreased for vesicles with a high mole fraction of phosphatidylserine. This is probably due to the aggregation of the vesicles at 10 mM $CaCl_2$. The use of larger vesicles did not result in a significant increase of the sites present.

It has been reported that the addition of $CaCl_2$ to small vesicles composed of pure phosphatidylserine results in the formation of structures known as scrolls (5). These have an apparent diameter of 2300 Å as determined by 90 ° light scattering (6). When EDTA is added large vesicles remain with a diameter of 2400 Å (6). Measurement of the changes in optical density in the presence of

$CaCl_2$ showed that the optical density for these large vesicles increased to the same level than the optical density for solutions of small vesicles. Therefore, it appears that these large vesicles again form scrolls.

The use of RVV-X for the measurement of binding parameters of factor X binding offers an advantage over other methods published thus far. It is easier than the method of Hummel & Dryer (8) and requires much less protein. The light scattering technique cannot be used at high $CaCl_2$ concentrations when vesicles with a high phosphatidylserine content are present. When RVV-X is used this offers no problem. It is then possible to determine the binding parameters of factor X at exactly the same conditions as are used in the kinetic experiments of factor X activation by factor IX_a in the presence of $CaCl_2$ and phospholipid (cf. chapter 5). In that case it is no longer of any significance whether the vesicles are single bilayer or not since K_d and the amount of sites can be determined using the same conditions.

For the inference of the binding parameters for factor X binding from the observed kinetics of factor X activation by factor IX_a in the presence of phospholipid and $CaCl_2$ (analous to ref.9) one needs the assumption that rates of factor X_a formation observed are a reflection of binding of the proteins to the phospholipid vesicles. However, it is of great advantage that an independent technique for the measurement of binding parameters can now be used to test this assumption.

ACKNOWLEDGEMENTS

Part of the chromogenic substrate S 2222 was kindly donated by AB Kabi. We thank Dr. E.M. Bevers, Mr. P. Confurius and Prof.Dr. R.F.A. Zwaal for stimulating discussions.

FOOTNOTES

*) Abbreviations used are: RVV-X, purified factor X activator from Russel's viper venom; S 2222, N-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-p-nitroanilide hydrochloride; f X, factor X; f X_a, factor X_a.

REFERENCES

1. Zwaal, R.F.A. (1978) *Biochim.Biophys.Acta* 515, 163-205
2. Nelsestuen, G.K. and Lim, T.K. (1977) *Biochemistry* 16, 4164-4171
3. Nelsestuen, G.K. and Broderius, M (1977) *Biochemistry* 16, 4172-4181
4. Bloom, J.W., Nesheim, M.E. and Mann, K.G. (1979) *Biochemistry* 18, 4419-4425
5. Papahadjopoulos, D., Vail, W.J., Jacobson, K. and Poste, G. (1975) *Biochim.Biophys.Acta* 394, 483-491
6. Day, E.P., Ho, J.T., Kunze Jr, R.K. and Sun, S.T. (1977) *Biochim.Biophys.Acta* 470, 503-508
7. Dombrose, F.A., Gitel, S.N., Zawalich, K. and Jackson, C.M. (1979) *J.Biol.Chem.* 254, 5027-5040
8. Hummel, J.P. and Dreyer, W.J. (1962) *Biochim.Biophys.Acta* 63, 530-532
9. Nesheim, M.E., Taswell, J.B. and Mann, K.G. (1979) *J.Biol.Chem.* 254, 10952-10962
10. Eisenthal, R. and Cornish-Bowden, A. (1974) *Biochem.J.* 139, 715-720
11. Kosow, D.P., Furie, B. and Forastieri, H. (1974) *Thromb.Res.* 4, 219-227
12. Lindhout, M.J., Kop-Klaassen, B.H.M. and Hemker H.C. (1978) *Biochim.Biophys.Acta* 533, 327-341
13. Jesty, J., Spencer, H.K. and Nemerson, Y (1974) *J.Biol.Chem.* 249, 5614-5622
14. Fujikawa, K., Titani, K. and Davie, E.W. (1975) *Proc.Natl. Acad.Sci.USA* 72, 3359-3363
15. Gerritsen, W.J., Verkley, A.J., Zwaal, R.F.A. and van Deenen, L.L.M. (1978) *Eur.J.Biochem.* 85, 255-261

CHAPTER 8

GENERAL DISCUSSION

The activation of factor X, both via the extrinsic and intrinsic pathway, and prothrombin are very similar.

The enzyme responsible for prothrombin activation in vivo is the serine protease factor X_a. Serine proteases are in general powerful enzymes. For small substrates k_{cat}'s have been reported in the order of 100 s^{-1} (1-3). However, prothrombin is a poor substrate for factor X_a. The K_m for prothrombin is well above the plasma concentration and the k_{cat} of the reaction is low (see Table I). Therefore, it is not surprising that under physiological conditions accessory components are required to increase the rates of prothrombin activation to a level sufficient to control haemostasis. Jobin and Esnouf (4) and Esmon et al. (5) showed that the relative rates of prothrombin activation are enormously enhanced by the presence of phospholipid, $CaCl_2$ and a protein co-factor (factor V_a).

Factor X activation via the intrinsic and extrinsic pathway is accomplished by respectively the serine proteases factor IX_a and factor VII_a. Factor X activation by factor IX_a is markedly enhanced by the presence of phospholipid, $CaCl_2$ and factor $VIII_a$ whereas the relative rate of factor X activation by factor VII_a is stimulated by the presence of $CaCl_2$ and tissue thromboplastin (6,7). Tissue thromboplastin is a complex of phospholipid and one or more protein co-factors (7).

The enhancement of the relative rates of activation of prothrombin and factor X in the presence of accessory components will be caused by a change of the kinetic parameters of these reactions. In Table I the kinetic data presented in this thesis on prothrombin and factor X activation are summarized together with recent data from literature. The changes of the kinetic parameters of prothrombin and factor X activation measured in the presence of accessory components explain the above mentioned rate enhancements. The protein co-factors factor V_a, factor $VIII_a$ and the protein component of thromboplastin have similar effects on the kinetic parameters. They cause a dramatic increase of the k_{cat} of their respective reactions. The effect of phospholipid in the three complexes is also similar. The presence of phospholipid causes a marked decrease of the K_m for prothrombin in the prothrombinase complex and of the K_m for factor X in both the intrinsic and extrinsic factor X activating complex.

Table I Kinetic constants of prothrombin and factor X activation

Enzyme	Substrate	Accessory components	K_m (μM)	k_{cat} (s^{-1})	Source
X_a	prothrombin	Ca^{2+}	84	0.011	1
X_a	,,	Ca^{2+},PL(7.5μM)	0.06	0.038	1
X_a	,,	Ca^{2+},PL(7.5μM),V_a	0.2	32.0	1
X_a	,,	Ca^{2+},PL(63μM)	0.63		2
X_a	,,	Ca^{2+},PL(11.5μM),V_a	1.03	35.0	3
IX_a	factor X	Ca^{2+}	181	1.75×10^{-4}	4
IX_a	,,	Ca^{2+},PL(10μM)	0.058	4.11×10^{-5}	4
IX_a	,,	Ca^{2+},PL(10μM),$VIII_a$	0.063	8.33	4
VII_a	,,	Ca^{2+},PL	4.87	3.95×10^{-4}	5
VII_a	,,	Ca^{2+},PL,tissue factor	0.45	1.15	5

1. Chapter 4 of this thesis.
2. PL vesicles were composed of a mixture of egg phosphatidylethanolamine and phosphatidylserine (Folch fraction III) in a ratio of 2/1;mole/mole. Taken from ref. 22.
3. PL vesicles were composed of a mixture of phosphatidylserine (Folch fraction III) and soybean phosphatidylcholine (25/75; mole/mole). Taken from ref. 19.
4. Chapter 5 of this thesis.
5. PL concentration was 0.75 mg/ml of a suspension of rabbit brain cephalin. Taken from ref. 8

The physiological significance of the changes of the kinetic parameters observed in the presence of accessory components is clear. Phospholipid is required to bring the K_m for prothrombin and factor X in the range of their respective plasma concentrations while the accessory protein components are required to increase the k_{cat} of the reactions to a level where sufficient activated clotting factors are formed to control haemostasis.

The role of factors V_a and $VIII_a$ in respectively prothrombin and factor X activation

In chapter 4 it was shown that factor V_a has little influence on the K_m for prothrombin but increases the k_{cat} of thrombin formation

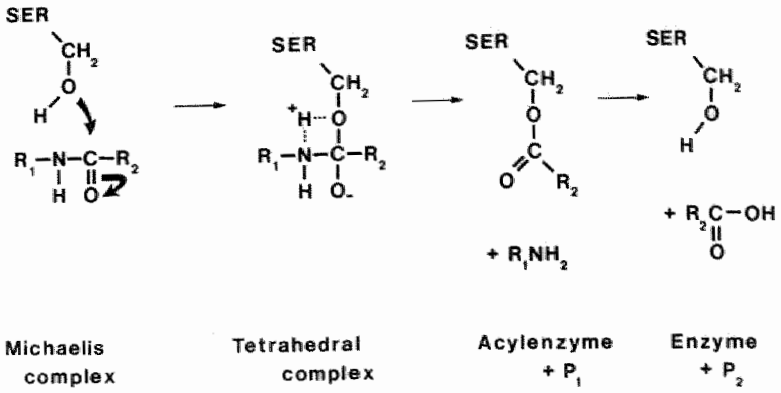


Fig. 1. Molecular mechanism of peptide bond splitting by serine proteases

In the first step the Michaelis complex is formed between the enzyme and the substrate through non-covalent interactions. In this complex the substrate is located in the active site pocket in such a way that the nucleophylic attack of the active site serine of the enzyme on the carbonyl group of the peptide bond to be split in the substrate is facilitated. After going through the tetrahedral intermediate the first product (P1) and the acylenzyme result. Upon hydrolisation of the acylenzyme the second product (P2) is liberated and the active site comes free again.

dramatically. In chapter 5 it was shown that factor $VIII_a$ has the same effect in factor X activation by factor IX_a. However, from this change in k_{cat} no conclusions can be drawn about the mode of action of these proteins in the mechanism of prothrombin and factor X activation. In order to be able to draw such conclusions more information about the molecular mechanism of the respective reactions is required.

Factor X_a and factor IX_a are highly homologous to the serine proteases trypsin and chymotrypsin. Therefore, it is likely that the mechanism of peptide bond cleavage in prothrombin and factor X will be similar to the general mechanism of peptide bond cleavage by serine proteases. The mechanism via which trypsin and chymotrypsin act upon their substrates is quite well understood (see ref. 9).

Fig. 1 shows the molecular changes occuring upon interaction of a serine protease with its substrate. The first step is the formation of a complex between the enzyme and the substrate via non-covalent

interactions (the so-called Michaelis complex). In all serine proteases of which the three-dimensional structure is known it has been found that the active site serine residue is hydrogen bonded to a histidine residue which in turn is hydrogen bonded to an aspartic acid residue. This line-up of SER-HIS-ASP, called the charge-relay system, is thought to greatly facilitate the next step occuring in the mechanism, i.e. the formation of a covalent intermediate (tetrahedral intermediate) through nucleophylic attack of the active site serine on the carbonyl group of the peptide bond to be split. The tetrahedral intermediate rapidly dissociates into the acyl-enzyme and the first product (P1) is liberated. The final step is the hydrolysis of the acyl-enzyme intermediate in which the second product (P2) is liberated and the active site comes free again.

From the work of the groups of Davie (7) and Nemerson (10) it can be concluded that the acyl-enzyme in factor X activation by factor IX_a is a covalent complex of factor IX_a and the activation peptide of factor X, i.e. the N-terminal part of the heavy chain up to and including the ARG 51 residue. Thus a reaction scheme for factor X activation by factor IX_a can be depicted as follows:

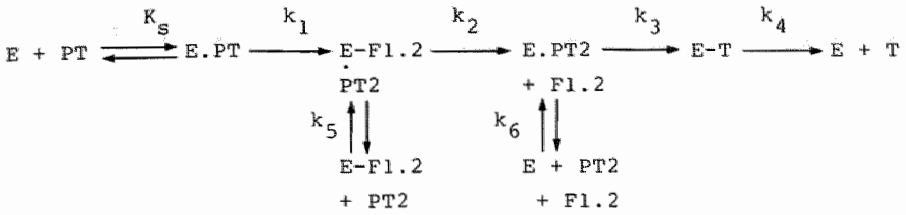
$$E + X \underset{}{\overset{K_s}{\rightleftharpoons}} E.X \xrightarrow{k_1} E\text{-}F \xrightarrow{k_2} E + F$$
$$+ X_a$$

Here E is the enzyme factor IX_a, F is the activation peptide and X_a is the product of the reaction.

The k_{cat} of an enzymatic reaction cannot be greater than any first order rate constant in the forward reaction pathway. Therefore, an increase in V_{max} can come about by increasing one or more of these forward rate constants or by the removal of an inhibitory reaction product. Since factor X activation in the absence of factor $VIII_a$ is linear in time and proportional to the amount of enzyme present, it is unlikely that inhibition by reaction products occurs. We conclude that factor $VIII_a$ increases the V_{max} of factor X_a formation by increasing one or more of the forward rate constants in the reaction pathway.

In prothrombin activation two peptide bonds have to be split by the enzyme factor X_a (cf. chapter 2). Although the order of peptide bond splitting is not known it is generally assumed that first prethrombin 2 is formed (liberating the activation peptide fragment 1.2) and that

subsequently prethrombin 2 is cleaved to produce thrombin. The general reaction scheme can be depicted as follows:



Prothrombin (PT) binds to factor X_a (E) and after going through the Michaelis complex and tetrahedral intermediate, the acyl-enzyme consisting of the activation peptide fragment 1.2 (F1.2) and factor X_a is formed. Before the next bond can be split the acylenzyme has to be hydrolised. The activation peptide dissociates from the enzyme and a new Michaelis complex between factor X_a and prethrombin 2 (PT2) can be formed whereupon thrombin is formed. This mechanism leaves ample possibility for the prethrombin 2 molecule to dissociate from the enzyme. Reassociation, which would offer a second chance to be converted to thrombin, is prevented by the excess amount of prothrombin competing for the same enzyme. Therefore, in this mechanism thrombin formation can be very slow compared to prethrombin 2 formation. In chapter 4 it was shown that in the absence of factor V_a the main end product of the reaction is not thrombin but prethrombin 2. Only small amounts of prethrombin 2 slip through and are converted to thrombin which is measured in the kinetic experiments. When factor V_a is present the pathway of activation shifts from one resulting in prethrombin 2 into one resulting in thrombin. We conclude that the presence of factor V_a prevents dissociation of prethrombin 2 from the enzyme. This can be achieved in two ways. Either the dissociation of prethrombin 2 is prevented directly by lowering the dissociation constant or indirectly by lowering the steady state concentration of the enzyme-prethrombin 2 complex, by increasing a limiting forward rate constant (k_2-k_4 in the reaction scheme). Direct prevention of dissociation of prethrombin 2 by factor V_a is not unlikely because it has been shown that factor V_a binds to the fragment 2 region of the prothrombin molecule and tight non-covalent interaction between fragment 1.2 and prethrombin 2 has been reported (11). We do no think that this can be the only mechanism involved. Since the k_{cat} in the presence of factor V_a is about 50 s^{-1}each forward rate constant must be at least 50 s^{-1}.

Table II Estimation of the rate of prethrombin 2 and thrombin formation by factor X_a in the presence of $CaCl_2$ and phospholipid

Incubation time minutes	Thrombin formed µmoles/ml[a]	µmoles/ml[b]	Prethrombin 2 formed µmoles/ml[c]
2	0.065×10^{-4}	-	0.7×10^{-4}
5	0.16×10^{-4}	0.2×10^{-4}	1.9×10^{-4}
10	0.34×10^{-4}	0.5×10^{-4}	3.1×10^{-4}
20	0.66×10^{-4}	0.88×10^{-4}	5.3×10^{-4}
30	0.78×10^{-4}	0.86×10^{-4}	6.2×10^{-4}

Estimated k_{cat} of thrombin formation 0.05 s^{-1} [a]; 0.075 s^{-1} b
k_{cat} of prethrombin 2 formation 0.45 s^{-1} c

Prothrombin (4 µM) was incubated with f X_a (1.2 pmoles/ml), phospholipid (75 µM) and $CaCl_2$ (30 mM) in a buffer containing 100 mM NaCl and 50 mM Tris-HCl at 37 °C at pH 7.5. After different time intervals samples were taken and assayed for thrombin using S 2238[a]. In order to get an estimate of the rate of prethrombin 2 formation, samples were taken and run on SDS-gels. Together with these gels, gels with known amounts of prethrombin 2 and active site titrated thrombin were prepared. After staining and destaining of the gels, they were scanned on a Gilford Model 250 spectrophotometer. The bands on the gels with known amounts of thrombin and prethrombin 2 were integrated and from a calibration curve the amounts of thrombin[b] and prethrombin 2[c] present in the activation mixture could be calculated.

If factor V_a only decreases the dissociation rate constant of prethrombin 2 and would not influence a forward rate constant these rate constants in the absence of factor V_a also have to be at least 50 s^{-1}. In the absence of factor V_a, however, we measure a k_{cat} of prethrombin 2 formation of about 0.5 s^{-1} and a k_{cat} of thrombin formation of about 0.05 s^{-1} (see Table II). This is in no way compatible with individual forward rate constants of 50 s^{-1} or more. We conclude that apart from a possible role in lowering the prethrombin 2 dissociation constant there must be at least one rate limiting forward rate constant in the activation pathway that is raised by factor V_a. This is, of course, in aggreement with the fact that factor $VIII_a$ exerts an analogous effect in factor X activation where only one peptide bond in the subtrate

has to be cleaved. The experimental evidence obtained with both complexes thus far allows no speculations on the localisation of this rate limiting step. A thorough kinetic study of the appearance and disappearance of intermediates and reaction products will be required to solve this question.

The role of phospholipid in prothrombin and factor X activation

It is generally accepted that the phospholipid bilayer exerts its coagulant activity when it is able to bind the proteins involved. Phospholipid bilayers stimulate coagulation only when they possess a net negative charge. Factors IX_a, X_a, X and prothrombin are thought to bind to the phospholipid bilayer via Ca^{2+}-bridges between the γ-carboxyglutamic acid residues of these proteins and the negatively charged phospholipid head groups. The factors V_a and $VIII_a$ presumably bind via more hydrophobic interaction (cf. chapter 2).

It is thought that under physiological conditions the blood platelet membrane providesthe phospholipid surface required for factor X and prothrombin activation. This procoagulant activity of the platelet has been referred to as platelet factor 3 (PF-3) activity. However, platelets have no significant amount of negatively charged phospholipids in the outer layer of the plasma membrane. To stimulate coagulation the negatively charged phospholipids which are present inside the platelet must become exposed.

Until a few years ago the postulated appearance of negatively charged phospholipids has been assumed to be identical to PF-3 activity. In recent years doubt has grown whether PF-3 is indeed identical to phospholipid. It has been reported that factor X_a binds with a high affinity to a specific receptor present on the platelet surface. It was demonstrated that this receptor is factor V_a bound to the outside of the platelet membrane (12-16). The binding site for factor V_a on the platelet is thought to be a specific receptor which, apart from a possible role of phospholipid, probably requires a protein component. Thus it is thought that the driving force bringing factor V_a and factor X_a together on the platelet surface is the interaction of factor V_a with a specific receptor (probably a protein) and of factor X_a with bound factor V_a. The receptor theory is supported by the findings of the group of Mann. They found that factor V_a binds more tightly to platelets than to vesicles composed of a mixture of phosphatidylserine (Folch fraction III) and soy-bean phosphatidylcholine (25/75;w/w) (17,18) and that factor X_a binds more tightly to phospho-

lipid vesicles when factor V_a is present (19).

The factor X_a thus bound to intact or released platelets via bound factor V_a can convert prothrombin at the same rate as factor X_a bound to a phospholipid surface of a mixture of phosphatidylserine and phosphatidylcholine in the presence of saturating amounts of factor V_a.

The question which remains is: where do phospholipids come into this picture? Are in addition to the receptors for factor V_a and factor X_a negatively charged phospholipids required for prothrombin and factor X activation under physiological conditions? We think the answer to this question is yes.

First, the platelet surface should play a role in factor X activation. However, it has not been reported that factor $VIII_a$ and factor IX_a bind to the platelet surface via a specific receptor. Second, it is not sufficient that factor V_a and factor X_a bind to the platelet surface. Interaction with prothrombin as well has to be taken into account. Thus far no evidence has been reported that prothrombin would bind better to platelets or phospholipid in the presence of factor V_a and factor X_a. Third, in the papers in which prothrombin activation by factor X_a bound to intact or released platelets was reported no corrections were made for platelet lysis (12-16). Thus small amounts of platelets may have been disrupted and this may have resulted in small amounts of negatively charged phospholipids to become available for prothrombin activation. Moreover, to control haemostasis PF-3 activity should become available. The total amount of thrombin produced with intact or thrombin activated platelets is low. When 100% PF-3 is available the rate of thrombin production is increased about a 100-fold. Therefore, 1% lysis could explain the reported prothrombin activation with intact or released platelets. Fourth, phosphatidylserine is demonstrable in the outer layer of the plasma membrane when platelets are stimulated by the combined action of thrombin and collagen (20,21). Since this was demonstrated to occur without disruption of the platelets this is presumably caused by a translocation of phosphatidylserine over the platelet membrane. Concomittant with the occurence of phosphatidylserine in the outer layer of the plasma membrane PF-3 activity appeared. It is unlikely that this occurs as a result of the platelet release reaction since it was shown that upon the separate action of collagen or thrombin, though stimulating the platelet release reaction, a small amount of PF-3 activity appeared which could be attributed completely to a small amount

of lysed platelets. Thus, prothrombin activation occurs only when phosphatidylserine appears in the outer layer of the plasma membrane. Therefore, although it is probable that platelets possess a specific receptor for factor V_a and that this bound factor V_a represents a specific receptor for factor X_a, we still think that negatively charged phospholipids are required to promote factor X and prothrombin activation.

Investigation of the kinetics of prothrombin and factor X activation in the absence and presence of phospholipid allows a first attempt to evaluate the role of phospholipid in these reactions.

Kosow and Orthner (22) carried out a kinetic study of prothrombin activation by factor X_a in the presence of $CaCl_2$ and phospholipid vesicles composed of a mixture of egg-yolk phosphatidylethanolamine and brain phosphatidylserine (2/1;mole/mole). They reported a K_m for prothrombin of 0.06 μM at 63 μM phospholipid and 10 mM $CaCl_2$. This is in reasonable aggreement with our results. The conclusions they arrived at for the mode of action of phospholipid are, however, in conflict with our results. They did not recognise that the K_m is dependent on the amount of phospholipid present and , from the small increase in V_{max} with increasing phospholipid concentrations, they concluded that the effect of phospholipid in prothrombin activation is due to an effect on V_{max}. They did not investigate the reaction in solution though. When the kinetics of thrombin and factor X activation in the presence and absence of phospholipid are compared to the kinetics of the reaction in solution (see Table II) it is clear that the presence of phospholipid enhances the relative rates of thrombin and factor X_a formation by causing a dramatic decrease in the K_m whereas the V_{max} is only slightly affected.

Two models can be put forward to qualitatively explain the effect of phospholipid in prothrombin and factor X activation. We will make a differentiation as strict as possible between these two in order to avoid unnecessary confusion.

The first model (model 1) is based on the assumption that it is the substrate bound to the phospholipid bilayer that is converted by the bound enzyme and that the free substrate does not participate in the reaction. The driving force bringing the substrate and enzyme together is the binding of the proteins to the phospholipid bilayer. The second model (model 2) is a model in which the bound enzyme acts upon the substrate <u>not</u> bound to the phospholipid bilayer.

Both models can qualitatively explain the observed effect of phospholipid on the kinetics of prothrombin and factor X activation. In model 1 in which it is assumed that the substrate bound to the bilayer is converted by the bound enzyme it is not necessary to assume that the kinetic parameters of the reaction change upon binding of the proteins to the phospholipid bilayer. Even if the kinetic parameters remain equal to those in solution a considerable decrease in K_m will be measured since the local substrate concentration is much higher than the bulk concentration. When the concentration of phospholipid is increased the density of the bound substrate decreases and more substrate has to be added to reach the same density on the phospholipid surface. Therefore, K_m apparent increases. In model 2 (free substrate converted by bound enzyme) the large decrease in apparent K_m can be explained when it is assumed that the binding affinity of the substrate for the catalytic site of the enzyme is changed as a result of the binding of the enzyme to the phospholipid bilayer. In that case the substrate binding sites of the phospholipid surface compete with the enzyme for the free substrate. When more phospholipid is added more substrate will be bound and more substrate has to be added to obtain the same level of free substrate. Therefore, K_m apparent increases.

The findings of the groups of Jackson and Majerus on factor X_a binding to platelets discussed above led Nelsestuen to propose yet another model for the role of phospholipid in prothrombin activation (23). Nelsestuen postulates that both factor X_a and prothrombin bind to an "active site" on the phospholipid surface, this active site being factor V_a bound to the bilayer. He states that the affinity of this active site (V_a-PL) for prothrombin "will be a composite of prothrombin-phospholipid and prothrombin-factor V_a interactions. This should represent a tighter binding than the simple prothrombin-membrane binding.' In view of the work presented in this thesis it is clear that this model cannot hold. The K_m for prothrombin in the presence of phospholipid either in the presence or in the absence of factor V_a is about the same. Therefore, the decrease in K_m by phospholipid cannot be caused by a specific phospholipid-factor V_a-prothrombin interaction.

In order to be able to explain the effect of phospholipid in prothrombin and factor X activation on a quantitative basis it is necessary to know the binding parameters of the substrate-phospholipid binding under the same conditions as are used in the kinetic experiments. It is then possible to convert the Lineweaver-Burk plots in which 1/(total substrate) was plotted against 1/v into Lineweaver-Burk plots of

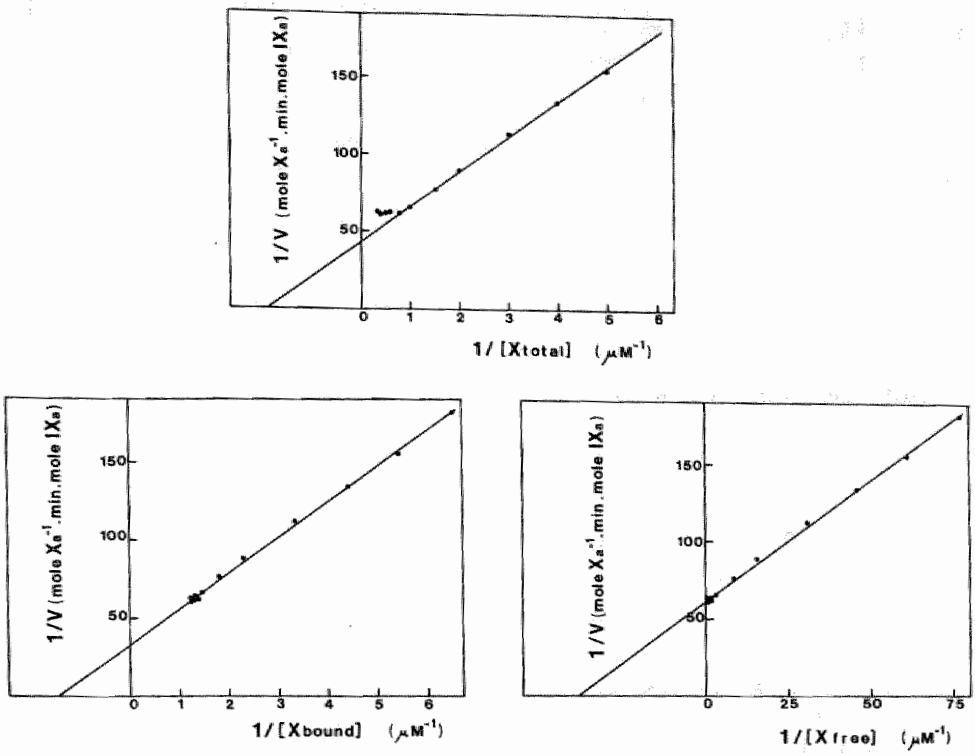


Fig. 2. Evaluation of the kinetics of factor X_a formation by factor IX_a in the presence of 100 μM phospholipid and 10 mM $CaCl_2$ in the two proposed models

Fig. 2A shows the Lineweaver-Burk plot in which 1/(total substrate) is plotted against 1/v. For experimental details see chapter 5. The amounts of bound and free factor X for each point in the Lineweaver-Burk plot were calculated using the binding parameters for factor X obtained in chapter 7. Lineweaver-Burk plots were constructed in which 1/(bound substrate) (B) and 1/(free substrate) (C) were plotted against 1/v. The K_m for bound factor X is 0.008 μM/μM phospholipid and the K_m obtained for free factor X as substrate is 0.03 μM (see also Table III).

1/(bound substrate) and 1/(free substrate) vs 1/v and to see whether the reaction obeys Michaelis-Menten kinetics with bound or with free substrate.

For factor X the binding parameters are known for the kind of vesicles used in the kinetic experiments described in chapter 5 (cf. chapter 7).

Table III Evaluation of the kinetic parameters of factor X_a formation by factor IX_a in the presence of phospholipid and $CaCl_2$ in the two proposed models

Phospholipid concentration	Total f X as substrate		Free f X as substrate		Bound f X as substrate	
	V_{max}[a]	K_m	V_{max}[a]	K_m	V_{max}[a]	K_m
(μM)		(μM)		(μM)		(μM)
10	0.0025	0.058	0.0021	0.021	0.0032	0.046
20	0.0058	0.139	0.0042	0.027	0.0079	0.144
50	0.0226	0.363	0.0161	0.035	0.0413	0.641
75	0.0219	0.409	0.0160	0.027	0.0302	0.549
100	0.0231	0.525	0.0164	0.026	0.0308	0.705
150	0.0295	0.822	0.0206	0.027	0.0400	1.140
200	0.474	1.830	0.0268	0.042	0.0631	2.485
300	0.0437	1.760	0.0300	0.032	0.0558	2.252

a. V_{max} is expressed as mole X_a.min^{-1}.mole IX_a^{-1}

The K_m calculated for free factor X as substrate is 0.03 μM and the K_m calculated for bound factor X as susbtrate is 0.008 μM/μM phospholipid.

Therefore, it was possible to calculate the amount of bound and free factor X for each point of the original Lineweaver-Burk plot (see chapter 5). In fig. 2 the different plots are shown. In both models the reaction appears to obey Michaelis-Menten kinetics. It will be clear that in both models the K_m expressed in bound or free substrate has to be independent of the phospholipid concentration. That this is the case is shown in Table III were the results of the calculations for the kinetic parameters at different phospholipid concentations in both models are given. In the model in which bound factor X is the substrate a K_m of 0.008 μM/μM phospholipid present is measured. In the model in which free factor X is the substrate the K_m is very low 0.025 μM.

The kinetic data obtained thus far allow no conclusions as to which of the two models is actually describing the real situation. However,

from the analysis of the data obtained thus far, predictions can be made for what to expect when phospholipid vesicles are used with a higher K_d than the vesicles used thus far. It will be expected that less substrate is bound by the phospholipid surface and therefore the free factor X concentrations will be higher. It will be easier to saturate the enzyme with substrate if free factor X is the substrate. On the other hand if bound factor X is the substrate it will require more factor X to saturate the binding sites. Thus it is expected that K_m apparent will increase if bound factor X is the substrate and that K_m apparent will decrease if free factor X is the substrate. Therefore, we are confident that in the near future it will be possible to determine which of the two models is valid.

REFERENCES

1. Zerner, B., Bond, R.P.M. and Bender, M.L. (1964) *J. Am. Chem. Soc.* 86, 3674-3683
2. Berezin, I.V., Kazanskaya, N.F. and Klyosov, A.A. (1971) *FEBS letters* 15, 121-127
3. Tans, G., Rosing, J., v. Dieijen, G.V. and Hemker, H.C. (1980) In The Regulation of Coagulation (Mann, K.G. and Taylor, F.B. Eds.) pp. 173-185
4. Jobin, F. and Esnouf, M.P. (1967) *Biochem. J.* 102, 666-674
5. Esmon, C.T., Owen, W.G. and Jackson, C.M. (1974) *J. Biol. Chem.* 249, 8045-8047
6. Hultin, M.B. and Nemerson, Y. (1987) *Blood* 52, 928-940
7. Fujikawa, K., Coan, M.H., Legaz, M.E. and Davie, E.W. (1974) *Biochemistry* 13, 5290-5299
8. Silverberg, S.A., Nemerson, Y. and Zur, M. (1977) *J. Biol. Chem.* 252, 8481-8488
9. Fersht, A.R. (1977) Enzyme structure and Mechanism, W.H. Freeman and Company, Reading and San Fransisco
10. Jesty, J., Spencer, A.K., Nakashima, Y., Nemerson, Y. and Konigsberg, W. (1975) *J. Biol. Chem.* 250, 4497-4504
11. Esmon, C.T. and Jackson, C.M. (1974) *J. Biol. Chem.* 249, 7791-7797
12. Miletich, J.P., Jackson, C.M. and Majerus, P.W. (1977) *Proc. Natl. Acad. Sci. USA* 74, 4033-4036
13. Miletich, J.P., Majerus, D.W. and Majerus, P.W. (1978) *J. Clin. Inv.* 62, 824-831
14. Miletich, J.P., Jackson, C.M. and Majerus, P.W. (1978) *J. Biol. Chem.* 253, 6908-6916

15.Miletich, J.P., Kane, W.H., Hofmann, S.L., Stanford, N. and Majerus, P.W. (1979) *Blood* 54, 1015-1022

16.Kane, W.H., Lindhout, M.J., Jackson, C.M. and Majerus, P.W. (1980) *J. Biol. Chem.* 255, 1170-1174

17.Bloom, J.W., Nesheim, M.E. and Mann, K.G. (1979) *Biochemistry* 18, 4419-4424

18.Tracy, P.B., Peterson, J.M., Nesheim, M.E., McDuffie, F.C. and Mann, K.G. (1979) *J. Biol. Chem.* 254,10354-10363

19.Nesheim, M.E. Taswell, J.B. and Mann, K.G. (1979) *J. Biol. Chem.* 254, 10952-10962

20.Zwaal, R.F.A., Rosing, J., Tans, G., Bevers, E.M. and Hemker, H.C. (1980) In The Regulation of Coagulation (Mannn, K.G. and Taylor, F.B. Eds.) pp. 95-112; Elsevier North Holland Inc, New York

21.Bevers, E.M., Comfurius, P., v. Rijn, J., Hemker, H.C. and Zwaal, R.F.A. (1980) submitted for publication

22.Kosow, D.P. and Orthner, C.L. (1979) *J. Biol. Chem.* 254, 9448-9452

23.Nelsestuen, G.L. (1980) In The Regulation of Coagulation (Mann, K.G. and Taylor, F.B. Eds.) Elsevier North Holland Inc. New York.

SUMMARY

In this thesis work is presented which concerns the role of protein-phospholipid interactions in coagulation. During coagulation a complicated chain of biochemical reactions occurs in which specific zymogens (clotting factors) are activated and which ultimately leads to the formation of thrombin. Thrombin converts fibrinogen into fibrin and the fibrin network, necessary for the arrest of bleeding from a wound, is formed.

In three reactions occuring in the so-called coagulation cascade phospholipid is thought to play a role. These reactions are factor X activation, both via the intrinsic and extrinsic pathway of coagulation, and prothrombin activation.

Phospholipid molecules spontaneously form lamellar structures when dispersed in an aqueous environment. These structures are known as bilayers. In these bilayers the phospholipid molecules are oriented with their chains towards each other and their head groups interact with the aqueous environment. Phospholipid bilayers show a peculiar feature. Above a certain temperature (transition temperature) the molecules in the bilayer can move freely in the plane of the bilayer. This is the so-called liquid crystalline state. Below the transition temperature diffusion of the molecules in the bilayer is hindered considerably. In chapter 3 it is shown that phospholipid bilayers in the liquid crystalline state promote coagulation much better than phospholipid bilayers in the solid gel state.

It was known already a long time that prothrombin activation by the enzyme factor X_a was stimulated enormously by the presence of $CaCl_2$, phospholipid and a protein co-factor, factor V_a. In chapter 4 it is shown that this stimulation is caused by important changes in the kinetic parameters of prothrombin activation. Phospholipids stimulate the reaction by bringing the K_m for the substrate prothrombin to a level which is about equal to the plasma concentration of prothrombin whereas factor V_a dramatically increases the k_{cat} of thrombin formation. Phospholipid and factor $VIII_a$ exert the same effect in factor X activation by factor IX_a as is shown by phospholipid and factor V_a in prothrombin activation (chapter 5).

In order to be able to express observed velocities in an enzymatic reaction in turnover rates of the enzyme it is necessary that the enzyme can be quantitated on a molecular basis. In chapter 6 it is

shown that factor IX_a can be quantitated via active site titration of the enzyme with p-nitrophenyl-p'-guanidinobenzoate.

The reactions investigated in this thesis are thought to take place on the phospholipid surface. Therefore, to appreciate the process going on on this surface it is essential that the parameters for the binding of the different proteins to the phospholipid bilayer are known. Chapter 7 describes a new method for the determination of the binding constant of factor X to phospholipid vesicles. The great advantage of this technique is that the binding parameters can be determined at exactly the same conditions as are used in the kinetic studies on factor X activation described in chapter 5. An additional advantage is that the method is relatively easy to perform.

In chapter 8 the findings presented in this thesis are discussed.

SAMENVATTING

In dit proefschrift worden de resultaten beschreven van onderzoek aan eiwit-lipide interakties in de bloedstolling. Stolling is o.a. het gevolg van een ingewikkelde reeks opeenvolgende biochemische reakties waarin eiwitten (stolfaktoren) die vrij in het bloed circuleren geaktiveerd worden. Deze reeks reakties leidt uiteindelijk tot de vorming van thrombine. Thrombine zet fibrinogeen om in fibrine en het fibrine netwerk, noodzakelijk voor bloedstelping, wordt gevormd.

Deze reeks opeenvolgende reakties wordt de stolcascade genoemd. In drie van deze reakties spelen fosfolipiden een rol. Dit zijn faktor X aktivering, langs de intrinsieke en extrinsieke weg, en prothrombine aktivering.

Wanneer fosfolipide molekulen in water opgenomen worden vormen ze spontaan lamellaire strukturen. Deze strukturen worden bilagen genoemd. De fosfolipide molekulen zijn in de bilaag met hun vetzuur staarten naar elkaar toe georienteerd. De kopgroepen vertonen interaktie met het water. Fosfolipide bilagen vertonen een speciale eigenschap. Boven een bepaalde temperatuur (overgangstemperatuur) kunnen de molekulen vrij bewegen in het vlak van de bilaag. De bilaag bevindt zich dan in de zgn. vloeibaar kristallijne fase. Beneden de overgangstemperatuur is de diffusie van fosfolipide molekulen in het vlak van de bilaag sterk gehinderd. In hoofdstuk 3 wordt aangetoond dat fosfolipide bilagen die zich in de vloeibaar kristallijne fase bevinden de stolling veel beter bevorderen dan bilagen die zich in de zgn. vaste fase bevinden.

Prothrombine wordt geaktiveerd door het enzym faktor X_a. Reeds lang was bekend dat deze reaktie enorm gestimuleerd wordt door $CaCl_2$, fosfolipiden en een eiwit co-faktor, faktor V_a. In hoofdstuk 4 worden experimenten beschreven die aantonen dat deze stimulering veroorzaakt wordt door belangrijke veranderingen in de kinetische parameters van prothrombine aktivering. Fosfolipiden stimuleren de reaktie doordat de K_m voor het substraat prothrombine verlaagd wordt tot een niveau dat ongeveer gelijk is aan de plasma concentratie van prothrombine. Faktor V_a stimuleert de reaktie door een sterke verhoging van de k_{cat} van thrombine vorming. In hoofdstuk 5 wordt aangetoond dat fosfolipiden en faktor $VIII_a$ eenzelfde effekt hebben op de aktivering van faktor X door faktor IX_a.

Wanneer men de snelheden van substraatomzetting wil uitdrukken in

hoeveelheden substraat omgezet per enzym is het noodzakelijk de hoeveelheden enzym aanwezig in het reaktievolume te kwantiteren. In hoofdstuk 6 wordt beschreven dat faktor IX_a concentraties gemeten kunnen worden door het enzym te titreren met p-nitrophenyl-p'-guanidinobenzoaat.

De reakties welke onderzocht zijn worden geacht plaats te vinden op het fosfolipide oppervlak. Om dit proces op het oppervlak te kunnen beschrijven en begrijpen is het noodzakelijk dat de bindingskonstanten van de eiwitten voor de binding aan de fosfolipide bilaag bekend zijn. In hoofdstuk 7 wordt een nieuwe methode geintroduceerd voor het meten van de binding van faktor X aan fosfolipide vesicles. Het voordeel van deze methode is dat ze relatief eenvoudig is en dat de bindingskonstanten gemeten kunnen worden onder dezelfde omstandigheden als gebruikt werden in de kinetische experimenten beschreven in hoofdstuk 5.

In hoofdstuk 8 wordt een algemene kritische beschouwing gegeven van de resultaten die in dit proefschrift gepresenteerd worden.

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 2 januari 1952 geboren te Maastricht. Na het doorlopen van de lagere school en de H.B.S.-B werd in 1969 een aanvang gemaakt met de studie scheikunde aan de Rijksuniversiteit te Groningen. Het kandidaatsexamen werd in 1973 afgelegd. Het doctoraalsexamen werd in 1976 afgelegd in de studierichting Fysische Chemie met als bijvak Informatietheorie.

Vanaf 1 mei 1977 is de schrijver werkzaam aan de Rijksuniversiteit Limburg te Maastricht bij de capaciteitsgroep Biochemie.